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## REDACTORES

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## The Rôle of the Reticulo-endothelial System in the Ripening of Reticulocytes.

By

ERIK JACOBSEN and CLAUS MUNK PLUM.

Received 26 Juli 1942.

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In a recent publication one of us (PLUM, 1942) has shown that the ripening of reticulocytes into mature red blood corpuscles is provoked by a hitherto unknown principle. This reticulocyte ripening principle is found in plasma and in various tissues and organs such as the spleen, the liver, and bone marrow. The chemical nature of this principle is still unknown, but in liver extracts we have found (1942) that it consists of at least two factors, one heatstable and one heatlabile, the former being identical with tyrosine.

When tyrosine is added to liver extract which contains some tyrosine the reticulocyte ripening activity is further increased. Thus the tyrosine content of the liver extract is not sufficient to engender a full activation of the ripening principle in liver extracts. The ripening principle in plasma cannot generally be further activated by tyrosine. As the tyrosine content of plasma is negligible the question arises as to whether the principles in plasma and in liver extracts are of a different chemical nature.

In the present paper we describe experiments showing that the reticulocyte ripening principle in plasma is probably either chemically or physically bound to tyrosine, and that the fixation of the tyrosine in the ripening principle seems to be connected with the function of the reticulo-endothelial system.



## Experimental:

### The chemical nature of the ripening principle in plasma.

(Ripening constant and ripening index.)

The testing of extracts for the reticulocyte ripening principle is described in detail elsewhere (PLUM, 1942) and will only briefly be outlined here. By repeated bleedings rabbits are brought into an anemic state which is accompanied by considerable reticulocytosis.

The red blood corpuscles from such an animal are separated from the plasma by means of centrifugation and washed with saline. When suspended in saline and incubated at body temperature the reticulocytes ripen very slowly, but when suspended in plasma or saline to which liver extract has been added the ripening proceeds much faster. The disappearance of the reticulocytes follows the monomolecular equation:

$$C = \frac{1}{t} \log. \frac{a}{(a - x)}$$

where  $t$  is the incubation time in hours,  $a$  the initial number of reticulocytes,  $(a - x)$  the number of reticulocytes found after incubation in the time  $t$ , and  $C$  a constant which is independent of  $t$ ,  $a$ , and  $(a - x)$  and it forms a measure for the velocity of the ripening process.

When  $k_s$  is the monomolecular constant of the ripening process in saline and  $k_h$  the corresponding constant of some suspending medium containing the ripening substance, the ripening effect of the latter,  $k$ , is expressed by the equation

$$k = k_h - k_s$$

$k$  here being defined as the *ripening constant*.

When using blood corpuscles from the same rabbit  $k$  is within certain limits proportional to the concentration of ripening substances.

Using reticulocytes from different rabbits, however, and the same concentration of ripening substance,  $k$  may vary considerably. It has therefore been necessary to introduce a standard in each experiment in order to get comparable results. This has led us to introduce the term *ripening index* which is defined thus:

$$\text{ripening index} = \frac{\text{ripening constant for the plasma examined}}{\text{ripening constant for ox plasma}}$$

Ox plasma having the highest content of ripening substances is, for reasons given elsewhere (PLUM 1942 b) fixed as having a ripening index of 1.00.

In most of the experiments we did not use ox plasma as the standard, but a 1 % dilution of a liver extract (Hepsol fortior) in saline solution which when compared with 11 different samples of ox plasma was found to have a ripening index of 0.97. The unit thus determined represented the average of 11 different samples of plasma.

Table 1.

*Plasma cannot be further activated by tyrosine.*

Reticulocytes incubated with	Monomolecular constant = C	Effect of tyrosine
Saline solution . . . . .	0.0108	—
Saline solution with 0.1 ‰ tyrosine . . . .	0.0125	0.0017
Rabbit plasma . . . . .	0.0224	—
Rabbit plasma with 0.1 ‰ tyrosine . . . .	0.0240	0.0016

Table 2.

*The tyrosine content of plasma is not sufficient to activate the ripening effect of a certain liver extract: H.*

Reticulocytes incubated with	Monomolecular constant = C	Effect of H and tyrosine
2.0 Saline solution . . . . .	0.0108	—
1.9 Saline solution + 0.1 H . . . . .	0.0203	0.0095
1.7 Saline solution + 0.1 H + 0.2 l ‰ tyrosine	0.0398	0.0290
1.6 Rabbit plasma + 0.4 Saline solution . . .	0.0224	—
1.6 Rabbit plasma + 0.1 H + 0.3 Saline solution . . . . .	0.0326	0.0102
1.6 Rabbit plasma + 0.1 H + 0.2 l ‰ tyrosine + 0.1 Saline solution . . . . .	0.0515	0.0291

Table 3.

*Effect of the treatment with acetone on the ability of the ripening substances in plasma to be activated by tyrosine.*

Incubated with	Monomolecular constant = C	Effect of tyrosine
Saline solution . . . . .	0.0100	—
1.8 Rabbit plasma + 0.2 Saline solution . . .	0.0269	—
1.8 Rabbit plasma + 0.2 l ‰ tyrosine . . .	0.0277	0.0008
1.8 plasma treated with acetone as described in the text + 0.2 Saline solution . . . .	0.0205	—
1.8 Plasma treated with acetone + 0.2 l ‰ tyrosine . . . . .	0.0289	0.0084

Table 4.

*Testing the functioning of the reticulo-endothelial system.*

Time after intravenous injection of 5 c.c. 1 % Congo red = t	extinction	$\frac{1}{(t-4)} \log. \frac{a}{(a-x)} = k$
4' . . . . .	0.374 (a)	—
20' . . . . .	0.292 (a — x)	0.0067
30' . . . . .	0.240 (a — x)	0.0074
60' . . . . .	0.154 (a — x)	0.0069

k: average = 0.0070

Table 5.

*Ripening index before and after blocking the reticulo-endothelial system.*

Rabbit plasma	without tyrosine	Ripening index		
		with 0.025 p. m. tyrosine	0.050 p. m. tyrosine	0.10 p. m. tyrosine
before blocking the reticulo- endothelial system . . . . .	0.74	—	0.80	—
24 hours after blocking the re- ticulo-endothelial system . .	0.44	0.59	0.79	0.83

In practice we determined the ripening index thus:

Blood from an anemic rabbit was taken in 1/4 vol. 3.8 % sodium citrate, the red blood cells were centrifuged off and washed with saline. The blood cells were then divided into portions which were suspended in (1) saline, (2) saline with 1 % liver extract (standard) and (3) the plasma to be examined. The portions were kept in a thermostat at 40° and their reticulocyte percentage was counted at the outset and after 2, 4, and 6 hours incubation. By means of the formulas given above the ripening constants of the standard and the plasma were calculated and thus the ripening index found from the formula:

$$\text{ripening index} = \frac{\text{ripening constant for plasma}}{\text{ripening constant for standard}} \times 0.97$$

Table 1 shows that the ripening principle in plasma cannot be further activated by tyrosine. Table 2 shows that the tyrosine content of plasma is too small to activate a liver preparation "H" which is considerably activated by tyrosine, and it shows furthermore that the activating effect of tyrosine is the same whether the ripening substance from liver is diluted with saline solution or plasma.

The ripening principle in plasma can, however, after rather mild chemical treatments be brought into a state in which it can be activated, e. g. treatment with acetone or absorption by floridin with elution changes the nature of the ripening substance in plasma in this respect. In table 3 an experiment with acetone treatment is given. 300 c.c. acetone was added to 100 c.c. rabbits plasma and then the precipitated protein centrifuged off. The acetone water phase was then evaporated under reduced pressure until the total volume was 25 c.c. Tyrosine has no effect on untreated plasma whereas it activates to a considerable degree the plasma precipitated by acetone.

We concluded therefore that the ripening principle in blood is linked to tyrosine or some tyrosinelike substance.

### The ripening principle and the reticulo-endothelial system.

One of us (PLUM) has shown that the spleen, the liver, and the bone marrow are the most rich in ripening substances after the

plasma. As the tissues mentioned all form part of the reticulo-endothelial system it is natural to assume some connection between the formation of the ripening principle and the reticulo-endothelial system. This assumption was confirmed in preliminary experiments with rabbits where splenectomy or the intravenous application of indian ink caused a considerable decrease in the ripening index.

In the experiment here described, we blocked the reticulo-endothelial system in some rabbits, followed its functioning and compared it with the quantity of ripening substance in the plasma.

*The blocking of the reticulo-endothelial system* was performed by splenectomy combined with an intravenous injection of 10 c.c. of 1 % trypan blue. It will be seen that this procedure completely blocks the reticulo-endothelial system for one or two days and then its function is gradually resumed.

*The functioning of the reticular-endothelial system* was tested by the Congo red method first introduced by ADLER and REIMANN (1925), with some modification on several points. The method as originally used by ADLER and REIMANN is given in every large textbook (e. g. in *Abderhalden's Handbuch*) and its description will therefore be omitted here. It rests on the fact that intravenously injected Congo red is absorbed by the reticulo-endothelial system and the greater the function of the latter the faster the Congo red disappears from the plasma. Most of the earlier investigators using the method estimated the colour of the plasma in a very primitive way by means of the Sahli apparatus. Moreover, the original method only used two colour estimations four, and thirty or sixty minutes after the injection, the ratio between the colours in the two plasma or serum samples were then applied as the measure of the functioning of the reticulo-endothelial system. In order to get a more accurate method we introduced some modifications. After several experiments we fixed our method as follows:

From one ear in a rabbit we took 0.2 c.c. of blood in a capillary pipette and transferred it to a centrifuge tube containing 2.00 c.c. of a 3.8 % solution of sodium citrate. Then 5 c.c. of 1 % Congo red in saline solution was injected into a vein in the other ear. 4, 20, 30, and 60 minutes after the injection blood samples of 0.2 c.c. were taken and similarly put into centrifuge tubes with 2.00 c.c. of sodium citrate solution. *All* the blood samples were then centrifuged at 3—4,000 revolutions p. m. for at least half an hour. If the

samples are centrifuged in less than 30 minutes the solutions will be opaque and cannot be read in the photometer.

After centrifugation the clear solutions were read off with the Pulfrich photometer (filter S 50) against the blood sample taken before the injection of Congo red. Generally the 2.5 mm. cuvettes were the most suitable.

The disappearance of the colour followed the monomolecular equation, the constant expressing the velocity of the disappearance of the Congo red in the blood and thus the functioning of the reticuloendothelial system.

Table 4 gives a typical determination. The monomolecular constant is calculated according to the formula:

$$k = \frac{1}{(t - 4)} \log. \frac{a}{(a - x)}$$

where  $a$  is the extinction found in the blood sample four minutes after the injection,  $(a - x)$  the extinction in the sample after the time  $t$ , counted in minutes. Intense functioning of the reticuloendothelial system gives high constants whereas weak functioning gives low constants. In ten normal rabbits tested in this way we found the constants between 0.0054 and 0.0070 averaging 0.0065.

#### **The functioning of the reticulo-endothelial system and the quantity of reticulocyte ripening substance in plasma.**

Our principal experiment was carried out on ten rabbits in the following way:

On each rabbit the ripening index of the plasma with and without the addition of tyrosine was determined once a week. Immediately afterwards the animal was tested with Congo red in the way described.

At various times after the first test the animals had their reticuloendothelial system blocked as described according to the following scheme: Rabbits 182 and 183, blocked the day after the first examination and re-examined on the 6th, 13th and 20th days after splenectomy and treatment with trypan blue. Rabbits 184 and 185 blocked the 3rd day, reexamination on the 4th, 11th and 18th days after splenectomy. Rabbits 187 and 188 blocked the 5th day, re-examination on the 2nd, 9th and 16th days after splenectomy. Rabbit 187 died before the experiment was finished.

To these were added two further animals of which No. 189 was

tested the day after the blocking and died shortly afterwards. No. 190 had its ripening index tested on the first and second days after the operation and trypan blue treatment. Nos. 177 and 178 served as controls and remained untreated to ensure that the weekly testing with Congo red did not have any influence on the quantity

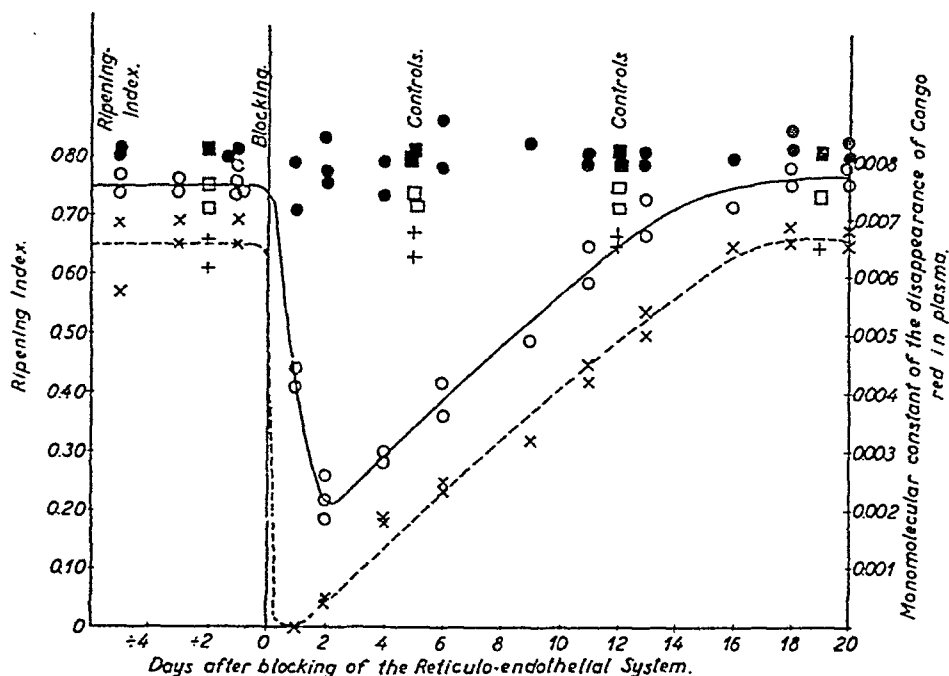


Fig. 1. Ripening index and function of the reticulo-endothelial system before and after the blocking of the latter.

× Functioning of the reticulo-endothelial system measured through the monomolecular constant of the disappearance of intravenously administered Congo red

† do of untreated controls

○ Ripening index without tyrosine

□ do do for untreated controls

● do with tyrosine

■ do do for untreated controls

of the ripening substances in the plasma. A couple of days after the trypan blue injection the plasma was intensely blue; this colour did not, however, interfere with either the reticulocyte ripening experiments or the Congo red tests. The filter used in the Pulfrich-photometer, S 50, did not absorb light passed through a trypan blue solution. The hemoglobin percentage of all the animals was followed, the red and white blood corpuscles and the reticulocytes were counted. The results of the experiments are put together in figures 1, 2, 3 and 4.

It will be seen that the blocking of the reticulo-endothelial system was effective. Actually the Congo red does not disappear from the blood. In a few days the functioning of the reticulo-endothelial system begins to increase and reaches its former value 16 to 18 days after the blocking. The ripening index too, reacts to blocking showing a considerable decrease, lowest on the second day, after which it increases, following closely the increase in the Congo red constants.

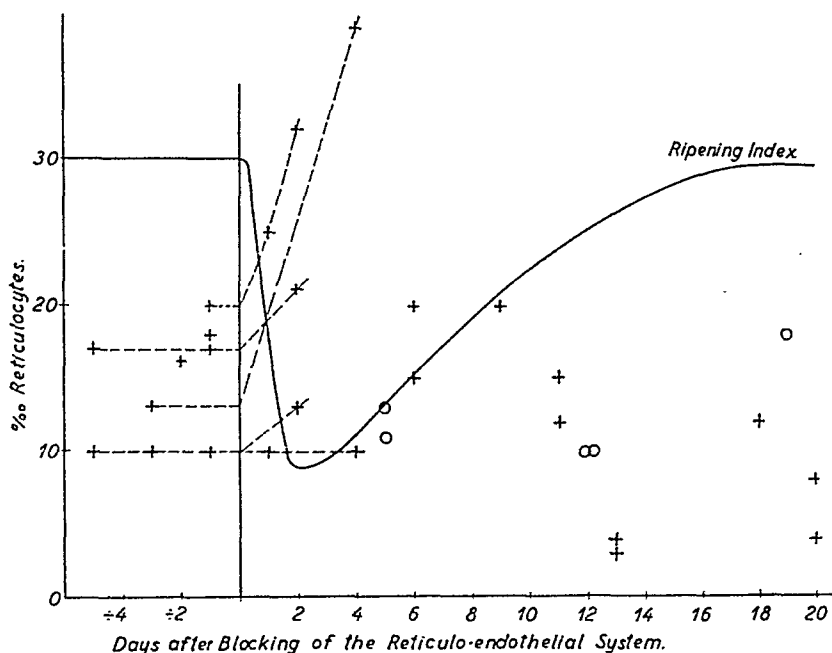


Fig. 2. Number of reticulocytes after blocking of the reticulo-endothelial system.  
 O untreated controls.

### Discussion.

One would expect the fall in the ripening index to be accompanied by an increase in reticulocytes. As a matter of fact this has been observed in a few animals during the first four days after blocking (fig. 2) but other animals show no alteration in this respect. As the number of the reticulocytes was not checked from day to day, all the animals may have shown this increase in reticulocytes which was not found in every case. Remarkable too is the strikingly low number of reticulocytes in some of the animals towards the end of the experiment. Fig. 3 shows slight anemia induced by the blocking and the leucocytes answer the blocking

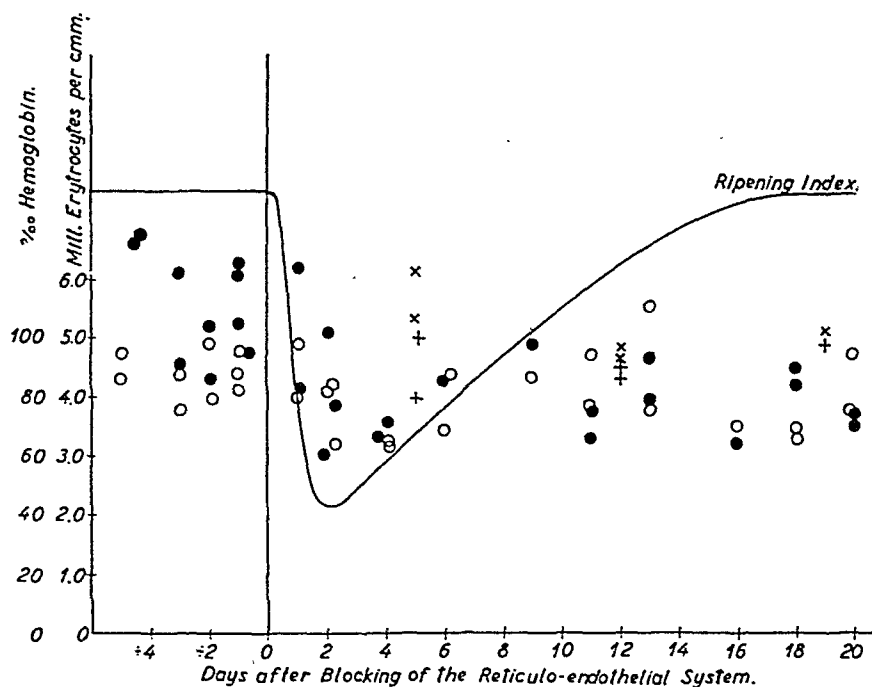


Fig. 3. Hemoglobin and red blood cells after blocking of the reticulo-endothelial system.

○ hemoglobin                    + hemoglobin  
● red blood cells                × red blood cells of untreated controls

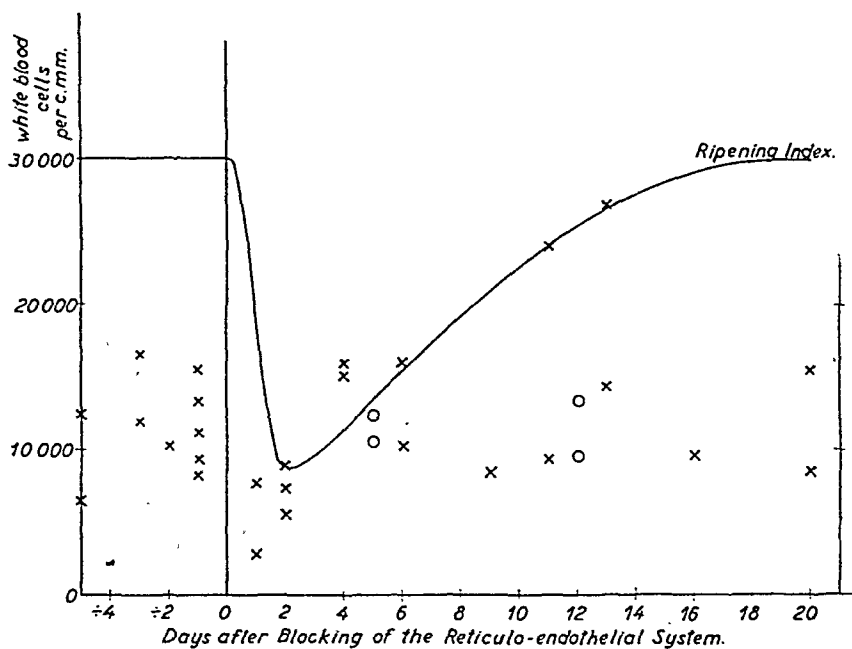


Fig. 4. White blood cells after blocking of the reticulo-endothelial system

○ Untreated controls.



with a transitory decrease in number (Fig. 4). The effects of the blocking of the reticulo-endothelial system on the hemopoiesis must, however, be rather complicated but as it is beyond the scope of the present paper to analyse this subject, further discussion will be omitted here in spite of its interest.

Very important is the effect of tyrosine. Unlike the case with normal plasma, tyrosine is able to activate the plasma with reduced ripening effectivity after blocking. Table 5 shows that 0.05 p. m. tyrosine is sufficient to give maximal activation. Not less

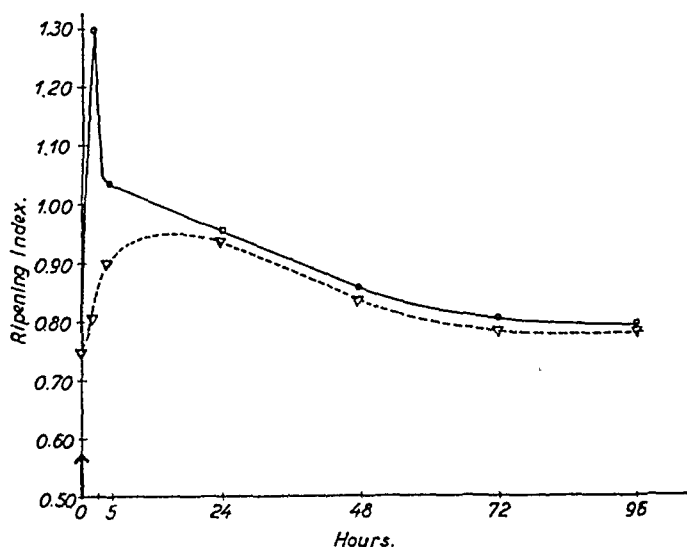


Fig. 5. Ripening index in rabbit plasma after injection of liver extract.

△ — — — △ = without addition of tyrosine  
 ○ — — — ○ = with addition of tyrosine.

remarkable is the fact that after adding tyrosine the very same ripening index is found as in normal plasma that cannot be activated.

This fact can only be interpreted to mean that the ripening substance in plasma after the blocking of the reticulo-endothelial system is deprived of something which tyrosine can substitute. Now, as demonstrated above, even chemical processes are sufficient to transform the ripening principle in plasma into a state capable of being activated by tyrosine. At present it may therefore almost certainly be assumed that the reticulocyte ripening principle in blood consists of two factors. An unknown factor must be linked to tyrosine or a substance closely related to tyrosine in order to form the ripening principle. The linkage between the un-

known factor and tyrosine can take place in vitro but this requires a concentration of tyrosine much higher than normally found in plasma. In vivo where the concentration of tyrosine is low the fixation of tyrosine to the unknown factor can only take place in cooperation with the reticulo-endothelial system. If the latter is blocked the linkage cannot occur and the tyrosine content of the plasma is too small to form the ripening principle. The unknown factor is formed elsewhere in the organism and its amount in plasma is not influenced by the reticulo-endothelial system.

That the organism has the power to transform a substance from a state in which it can be activated by tyrosine into a state in which it cannot be so activated, is clearly shown in an experiment made by CLAUS MUNK PLUM and RUTH PLUM. After an initial determination of the ripening index in plasma, a rabbit was given an intramuscular injection of 5 c.c. Hepsol fortior, a commercial liver extract containing a considerable amount of ripening substances able to be activated by tyrosine. At intervals after the injection the ripening index in plasma was determined without and with the addition of tyrosine. As seen in fig. 5 the ripening index in plasma increases. During the first hours after the injection the plasma can be activated to a rather considerable degree, but later on when the ripening index has reached its maximum, tyrosine has no further effect.

### Summary.

We briefly describe a method of estimating the amount of reticulocyte ripening principle, present in liver extracts and in plasma.

Unlike liver extracts the reticulocyte ripening principle in plasma cannot be further activated by tyrosine. The treatment of plasma with acetone for example transforms the ripening principle into a state in which it can be activated.

By means of splenectomy and the intravenous injection of trypan blue we blocked the reticulo-endothelial system in rabbits.

The effect of the blocking was controlled by a modification of the Congo red method, the colour of the plasma being measured in the Pulfrich photometer.

After blocking the reticulo-endothelial system the amount of the ripening principle in plasma first decreases considerably and then increases slowly, following closely the recovery of the reticulo-

endothelial system. The diminished quantity of ripening substance can be activated by tyrosine and this obtaining the same effect as before the blocking of the reticulo-endothelial system. These findings make it seem probable that tyrosine and an unknown substance are linked together in the reticulo-endothelial system and thus form the reticulocyte ripening principle found in plasma.

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## Antithrombin and Heparin.

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At the discovery of the blood clotting inhibiting substance heparin, HOWELL found that heparin itself is without any influence on the clotting of fibrinogen by thrombin when purified solutions are used (HOWELL and HOLT (1918), HOWELL (1925)). The establishment of this property requires the presence of some unknown components from plasma. Heparin thus is only an "antithrombin" in connection with these substances. The nature of the said components is still unknown, but according to QUICK (1938) it is the normal antithrombin which by addition of heparin is increased in potency. The problem is discussed by WÖHLISCH (1940).

Through our investigations on the relation between heparin and antithrombin we have come to a different conclusion, however, as we find that the normal antithrombin present in plasma cannot be activated further by heparin. The activation is therefore due to some other component present in plasma (ASTRUP and DARLING (1941 b)).

In order to distinguish between the normal antithrombin and the antithrombin formed from heparin, we have chosen the term "*thrombin inhibitor*" for the last-mentioned substance and retained the name *antithrombin* for the normal antithrombic properties of plasma and serum, as they are well known from older investigations and thus have a priority. The component from plasma which must be present for the formation of thrombin inhibitor is called *thrombin coinhibitor*.

The problem then arises to find out the nature of the coinhibitor and its relation to heparin. A further question is the pro-

properties of the thrombin inhibitor formed and its relation to the normal antithrombin. Experiments concerning these problems are presented in this paper.

## Experimental.

Throughout this work oxalated ox plasma was used and the results cannot be directly compared with results obtained with other plasmas, as there seem to be great differences between the properties of thrombin coinhibitor from plasmas of various species.

Thrombin was prepared as before (ASTRUP and DARLING (1940, 1941 a)), but most of it was obtained from "Løvens kemiske Fabrik", Copenhagen. The heparin, also obtained from "Løvens kemiske Fabrik", showed the activity  $k = 4-5$  (cf. ASTRUP and BEHRNTS JENSEN (1938 a, b), ASTRUP (1938)). Fibrinogen was prepared according to ASTRUP and DARLING (1942 a). As a rule it contained only negligible amounts of coinhibitor; if the content was found to be too large, it was discarded. The measurements of antithrombic power were carried out in principle as described in a preceding paper (ASTRUP and DARLING (1942 b)).

### 1. Thrombin Coinhibitor in Serum and Plasma.

HOWELL (1925), working with cat and dog blood, and QUICK (1938), working with rabbit blood, both found that the addition of heparin to serum obtained from these species gives rise to the formation of a powerful antithrombic substance.

According to our experiments, however, this does not apply to ox serum. The serum was prepared from oxalated ox plasma by one of the following three methods: 1) Recalcification of the plasma. 2) Removal of fibrinogen by addition of a minor amount of thrombin. 3) Denaturation of fibrinogen by heating to  $56^{\circ}$  in a water bath for 3 minutes. None of the sera prepared gave any increased content of antithrombic substances after addition of heparin. On the contrary, it was very often found that the antithrombin content was slightly decreased after the addition. For instance, a serum having originally 120 antithrombin units (A. T. U.) per ml showed a content of 100 A. T. U. per ml after addition of 1 mg of heparin per ml of serum. Even this amount of heparin was thus incapable of producing any antithrombic material in the serum used. Why the antithrombin content is slightly decreased is not known, but it is possibly due to a greater precipitability of the serum proteins in the presence of heparin

(FISCHER 1935) which may influence the formation and precipitation of fibrin and thus the clotting time.

The experiments cited show definitely that it is not the normal antithrombin present in plasma and serum which is increased in potency by the addition of heparin.

As the antithrombic potency of oxalated ox plasma measured by our usual method is increased by addition of heparin to over 200 A. T. U. per ml, the fate of the thrombin coinhibitor during the clotting process of such plasma is investigated.

The difficulty here arises that it is necessary to omit the incubation for 15 minutes at 37°, which otherwise must be carried out to obtain full saturation of the normal antithrombin of plasma and serum (ASTRUP and DARLING (1942 b)). Fortunately the thrombin inhibitor formed from heparin seems to react more readily with thrombin than the normal antithrombin itself does, so that by an immediate measurement without incubation a large fraction of the coinhibitor can be demonstrated. The determinations of course cannot be quantitative, but illustrates the progress of the reaction. The experiment is performed as follows:

In a tube 0.20 ml of plasma and 0.2 ml of a 1 per cent heparin solution are mixed, and then 1 ml of a thrombin solution is added containing 40 T. U. per ml. A clot of fibrin is formed instantaneously and the thrombin content of the remaining solution is immediately measured by placing 0.10 ml in a clotting tube and adding 1.0 ml of fibrinogen solution. A control is made by diluting 1.0 ml thrombin solution with 0.4 ml of physiological NaCl.

Then a sample of plasma is clotted by addition of a trace of thrombin, so that it clots in less than one minute. After standing for 2, 5 and 10 minutes respectively 0.20 ml of the serum formed is mixed with 0.20 ml of the heparin solution, 1.0 ml thrombin solution is added and the mixture immediately tested for thrombin content. The results are shown in Table 1.

Table 1.

Time Min.	T. U. found	A. T. U. found	A. T. U. per ml plasma	Coinhibitor per cent found
0 . . . . .	27	13	65	100
2 . . . . .	35	5	25	38
5 . . . . .	38	2	10	15
10 . . . . .	40	0	0	0

From Table 1 it is seen that already after 10 minutes the coinhibitor present in the plasma is completely destroyed. The anti-

thrombin present in serum or plasma seems not to take part in the inactivation when the measurements are carried out immediately without incubation. The destruction of the thrombin coinhibitor proceeds during the first minutes after the coagulation and very rapidly.

It is rather remarkable that the thrombin coinhibitor is such an unstable substance, and perhaps this fact explains some of the conflicting opinions of various authors.

## 2. Fractionation of Plasma.

It was then tried to divide plasma into fractions by means of ammonium sulphate in order to obtain some information about the nature of the coinhibitor.

From ox Bordet-plasma the fractions A, B and C are prepared as described in the investigations on antithrombin (ASTRUP and DARLING (1942 b)). The antithrombin content of these fractions are determined as described in the paper mentioned. The solutions are then incubated with heparin whereupon the total content of antithrombic material is determined. The increase is taken as a measure for the content of thrombin inhibitor and, thus, for the content of thrombin coinhibitor in the fraction used.

Of the different protein solutions 1.0 ml is incubated with 1.0 ml of a 0.1 per cent heparin solution for 10 minutes. Then 0.4 ml of the mixture is incubated with 1.0 ml of a thrombin solution for 15 minutes at 37° as usual. The thrombin content is measured as before and the content of antithrombin units per ml of protein solution determined. The results of such an experiment are shown in Table 2.

Table 2.

Fraction	Original A. T. U. per ml	Total A. T. U. per ml	Increase A. T. U. per ml	Original A. T. U. per mg N	Increase A. T. U. per mg N
A . . . . .	23	66	43	3.5	6.5
B . . . . .	0	48	48	0.0	10.0
C . . . . .	65	68	3	12.0	0.6

Fraction A is a globulin fraction while B and C are albumin fractions. Of these, C is the most soluble. As it is seen from Table 2, fraction C, in accordance with earlier investigations (ASTRUP and DARLING (1942 b)) showed the highest content of antithrombin, while fraction B is practically inactive. On addition of heparin the potency of C is practically unchanged, while fraction B now contains a powerful antithrombic substance.

The experiments thus confirm the preceding results. The normal antithrombin is not further activated by heparin, but the increased potency is due to the transformation of a substance which in itself is not an antithrombin. While the normal antithrombin belongs to the more soluble albumins, the new thrombin coinhibitor belongs to the lesser soluble albumins. Also the globulin fraction A can be activated by heparin which possibly is due to components from fraction B carried down with the precipitation of the globulin. The fractions are made from Bordet-plasma, which shows that treatment with tricalcium phosphate does not destroy the coinhibitor.

### 3. The Dissociation of Thrombin Inhibitor.

As already mentioned the fibrinogen often contains a trace of coinhibitor which is possibly due to its property as a less soluble albumin, as this furthers coprecipitation with the fibrinogen just as with the globulin fraction A. For the measurements such fibrinogen preparations must be discarded, but they may be used for investigating the properties of the coinhibitor. In the following experiment the influence of increasing amounts of heparin on the clotting of such a fibrinogen is investigated.

To a series of tubes containing 3.0 ml of a fibrinogen solution an amount ranging from 0.05 to 3.00 ml of a 0.1 per cent heparin solution is added; then the tubes are filled up with physiological NaCl to a volume of 6.0 ml. After incubation for 5 minutes at room temperature 1.0 ml of every sample is clotted by adding it to 0.10 ml of a thrombin solution in a clotting tube as usual. From the clotting time of the sample compared with the clotting time of a corresponding control solution of fibrinogen without heparin content, the amount of thrombin units active in the clotting process is found. From this the amount neutralized by the resulting thrombin inhibitor is calculated. The thrombin solution employed contained 20 T. U. per ml, so that when 0.10 ml is used, the clotting is carried out with 2.0 T. U. for every ml of fibrinogen mixture. The amount of thrombin units bound is shown in Fig. 1 as ordinate against the amount of heparin contained in one ml of the mixture as abscissa.

From Fig. 1 it is seen that the curve shows close resemblance to a curve for a dissociable compound in which the dissociation is forced in one direction by the addition of one of the components. Complete saturation of the coinhibitor is obtained only with addition of a large excess of heparin. In reaching this point there is in



1 ml of the mixture formed an amount of thrombin inhibitor which can inactivate 0.85 units of thrombin.

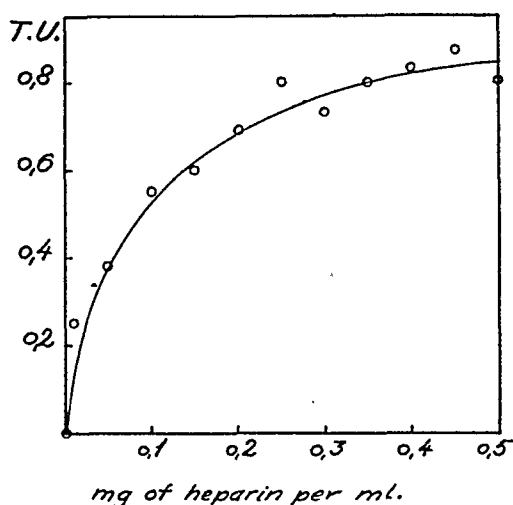


Fig. 1. Units of thrombin (T. U.) inactivated by a fibrinogen solution containing increasing amounts of heparin.

The thrombin inhibitor therefore seems to be a dissociable compound, while in the case of the normal antithrombin no evidence was found of this. Especially as normal antithrombin is not increased in potency on addition of heparin, it cannot be identical with the thrombin inhibitor formed from heparin. Otherwise the addition of heparin to the dissociated thrombin inhibitor would cause an increase in potency.

In a series of tubes 0.50 ml of solution B is placed. Increasing amounts of heparin are added, and then physiological NaCl to the total volume of 1.0 ml. The amounts of heparin range from 0.1 to 5 mg per sample, that is from 0.2 to 10 mg per ml of solution B. The mixtures are left standing for 15 minutes at room temperature. Then 1.0 ml of a thrombin solution containing 41 T. U. per ml is added to every sample. After incubation for 15 minutes at 37° the thrombin contained in the mixture is determined as usual. The thrombin content found is subtracted from the original value, thus giving the amount of thrombin inactivated in every sample. From this the amount of thrombin, inactivated per ml of solution B, is calculated by multiplying by two. The result is presented graphically in Fig. 2. The amount of heparin added per ml of solution B is abscissa, and the corresponding number of thrombin units inactivated is ordinate.

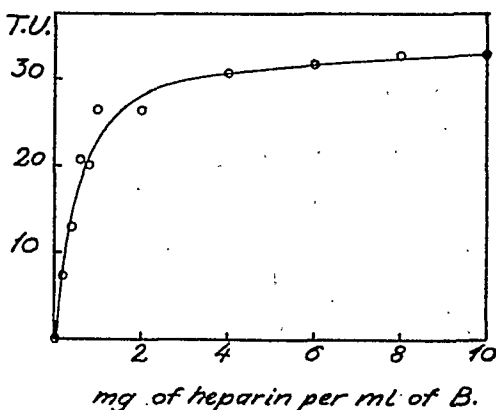


Fig. 2. Units of thrombin inactivated by a solution of thrombin coinhibitor containing increasing amounts of heparin.

The curve is quite similar to Fig. 1 and shows also evidence of a dissociation. Saturation degree is obtained only with a large excess of heparin, and a maximum of 32.8 T. U. is then inactivated per ml of solution B.

There seems to be no doubt about the thrombin inhibitor being a dissociable compound resulting from combination of heparin and coinhibitor. The relations, however, are not quite simple, as we are dealing not with a single process but with two consecutive reactions. The first reaction concerns the formation of the thrombin inhibitor from heparin and coinhibitor. In the second reaction the thrombin inhibitor formed reacts with the thrombin added and inactivates some of it. Obviously it is impossible to investigate the first reaction alone, as only the amount of thrombin removed by the second reaction is a measure for the thrombin inhibitor formed.

It is conceivable, however, that both these reactions imply dissociations, and that the inactive compound of thrombin and its inhibitor is dissociable.

If the inactive compound of thrombin and thrombin inhibitor was undissociated, the reaction on the addition of an excess of thrombin would proceed until all the thrombin inhibitor was used. Consequently the formation of thrombin inhibitor from heparin and coinhibitor would be forced in one direction until one of the two components was exhausted. The amount of thrombin inhibitor formed under these circumstances, *i. e.* in the presence of an excess of thrombin would thus be proportional to the amount of either heparin or coinhibitor. This, however, is not the case, as shown by the curves.

If, on the other hand, the inactive thrombin compound formed was dissociable and the thrombin inhibitor itself was undissociable, then the formation of the thrombin inhibitor would proceed until either heparin or coinhibitor was used up, regardless of the presence or absence of thrombin. The reaction between thrombin inhibitor and thrombin would therefore be independent of the presence of a larger or smaller excess of heparin. It is not possible *a priori* from the curves to see, if the heparin is in excess as such or in combined form as inhibitor, as in both cases the results will indicate a dissociation due to the dissociated compound between thrombin and its inhibitor. The curves, however, show that in order to force the reaction to saturation it is necessary to use an amount of heparin, which otherwise would have resulted in the formation of many times the amount of antithrombic material now formed. It seems likely therefore, that both the substances formed are dissociable compounds (see also the studies on heat inactivation below).

Both the reactions therefore seem to be dissociation processes, and they can be described by the following equations  $A_1$  and  $A_2$ :

$A_1$ : Heparin + thrombin coinhibitor  $\rightleftharpoons$  thrombin inhibitor.

$A_2$ : Thrombin inhibitor + thrombin  $\rightleftharpoons$  inactive thrombin compound.

As the reaction between thrombin and the normal antithrombin in plasma and serum shows no sign of being a reciprocal reaction under the conditions investigated, the process can be described by the following equation  $B$ :

$B$ : Antithrombin + thrombin  $\longrightarrow$  metathrombin.

In this equation the term metathrombin is used as the most common designation for the combination between antithrombin and thrombin, but it must be remembered that the nature and properties of metathrombin have not yet been fully elucidated.

Thrombin can be destroyed in plasma by two independent reactions.

#### 4. Investigations on Heat Inactivation.

In order to obtain a fuller knowledge of the nature of antithrombin, thrombin inhibitor and thrombin coinhibitor, the inactivation of these substances by heating was studied.

As *antithrombin* a fraction C from ox-plasma, prepared as described above, was used. It contained only negligible amounts of thrombin coinhibitor. Samples are put into tubes which are then placed in a water bath at various temperatures. At fixed intervals a sample is removed and placed in ice-water whereupon the antithrombin content is determined after our usual method. At both 100° and 75° the antithrombin is fully destroyed after only half a minute. At 65° the destruction is less rapid, and even slower at 56°. At 40° antithrombin is quite stable. The results obtained with the remaining potency calculated in per cent of the original potency are shown in Fig. 3.

As *thrombin coinhibitor* a corresponding fraction B containing no antithrombin is used. After treating the samples at different temperatures, 0.40 ml of a 1 per cent heparin solution is added to 0.60 ml of the solution. The mixture is then incubated with 1.0 ml thrombin solution containing 41 T. U. per ml at 37° for 15 minutes, and the thrombin content is determined as usual. From

the number of thrombin units removed the amount of antithrombic material is calculated. The results are shown in Fig. 4. The potency is calculated in per cent of the original strength.

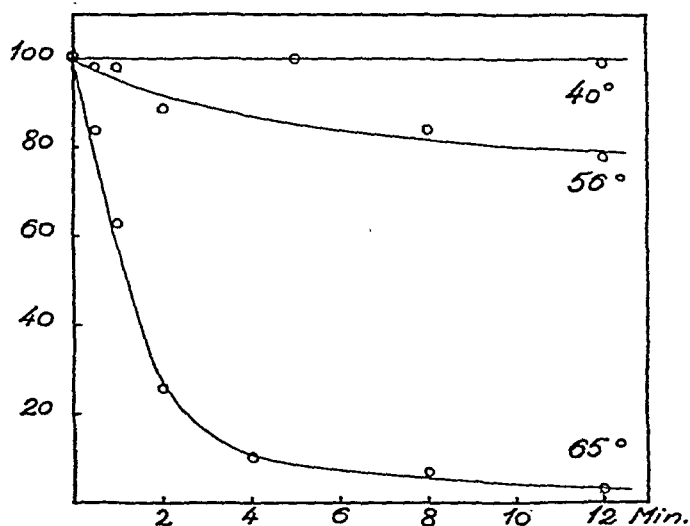


Fig. 3. Inactivation of antithrombin by heating.

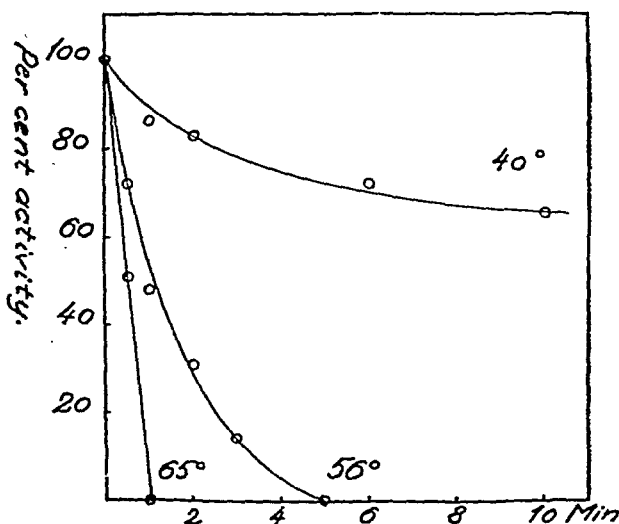


Fig. 4. Inactivation of thrombin coinhibitor by heating.

As it is seen from Fig. 4 the thrombin coinhibitor is a very unstable substance. It is very rapidly inactivated by heating, just as it is rapidly destroyed during the clotting process, as shown earlier in this paper. Already after 5 minutes at 56° it is fully destroyed, and even at 40° the inactivation proceeds with con

siderable velocity. This instability is quite in contrast to the greater resistance of the antithrombin.

For studies on the inactivation of *thrombin inhibitor*, 4 volumes of a 1 per cent heparin solution are added to 6 volumes of a B-fraction (coinhibitor containing no antithrombin). Samples of this mixture are treated at different temperatures as before. To 1.0 ml of every sample is then added 1.0 ml of a thrombin solution containing 41 T. U. per ml. The mixture is incubated as usual and the amount of antithrombic material determined. The results are shown in the usual manner in Fig. 5.

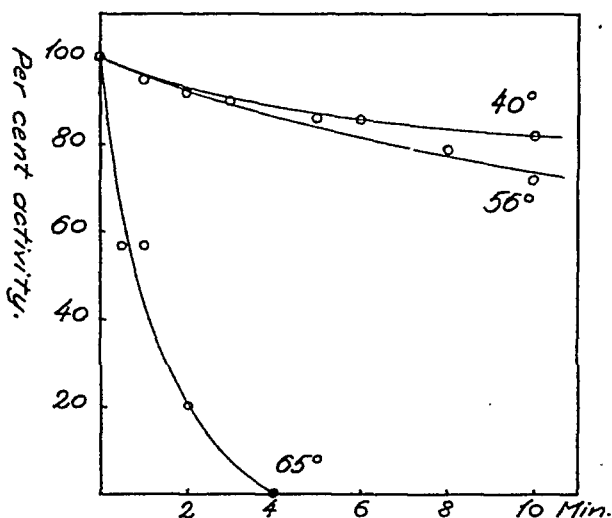


Fig. 5. Inactivation of thrombin inhibitor by heating.

From Fig. 5 it is seen that heparin greatly increases the stability of the coinhibitor, so that the resulting thrombin inhibitor is much more stable than its precursor. On comparison with the curves for antithrombin (Fig. 3) it is seen that the thrombin inhibitor formed is almost as stable against heating as the normal antithrombin. Yet, there is a difference which is particularly pronounced at 40°. At this temperature the antithrombin is quite stable, while the thrombin inhibitor slowly decreases in activity, though not so rapidly as the coinhibitor does. It is easy to explain this decreasing activity as due to dissociation of the thrombin inhibitor with a subsequent inactivation of the very instable free coinhibitor, by which process the dissociation is again furthered, resulting in the formation of a new lot of free coinhibitor, and so on. The experiments with inactivation by heating thus confirm

both the non-identity of antithrombin and thrombin inhibitor and the dissociability of the last-mentioned substance.

### 5. Antithrombin and Heparin.

As described in the first part of this work, we could not find any evidence for the presence of a coinhibitor in serum prepared either by clotting or by heating. With the preparation of the antithrombin-containing fraction C from plasma it is possible to investigate this further, as any trace of coinhibitor present can be destroyed without interfering much with the antithrombin by heating for a few minutes to 56°.

To 2.5 ml of an unheated solution of fraction C containing 70 A. T. U. per ml is added 0.5 ml of a 1 per cent heparin solution. After standing for 15 minutes the content of antithrombic material is again measured. It is found to be 76 A. T. U. per ml of C; The increase is 6 A. T. U., corresponding to 8.6 per cent.

A sample of C is now placed in a water-bath at 56° for 10 minutes. The content is then found (without heparin) to be 58 A. T. U. per ml *i. e.* 83 per cent of the original amount.

To samples of the heated C-solution are now added various amounts of heparin, ranging from 2 to 10 mg per cent of C-solution. In none of these samples was it possible to show any formation of thrombin inhibitor, as all the determinations were between 57 and 58 A. T. U. per ml.

It is thus confirmed that the antithrombin cannot be activated by heparin and therefore can be neither the coinhibitor nor the thrombin inhibitor, which also must be increased in potency due to the dissociation.

### Discussion.

As already mentioned, our results obtained so far are not quite concordant with the results of earlier authors. We have confirmed that the antithrombic action of heparin requires the presence of an unknown component of plasma, but regarding the properties of this component there is no good agreement. By earlier methods it has not been possible to obtain a quantitative measurement of the antithrombic substance resulting from heparin. In our investigations we have used the same principles as in our method for determination of the normal antithrombin (ASTRUP and DARLING (1942 b)). Unfortunately this method gives not quite satisfactory results with the antithrombin resulting from heparin (thrombin inhibitor). The curves obtained are not always

straight lines. This is true especially for ox plasma while it applies in a much lesser degree to rabbit and human plasma. Probably these difficulties arise from dissociation of the compound. Due to these circumstances, moreover, our method is not always quite quantitative; nevertheless, it yet seems to be the most satisfactory method used for the investigation of heparin antithrombin.

The most definite difference between our results and earlier experiences concerns the stability of the thrombin coinhibitor. While other investigators have found this substance present both in plasma and in serum, it was impossible for us to obtain evidence of its presence in *serum*, regardless of the method of preparation. We have found it to be a very thermolabile substance which is inactivated already after 5 minutes heating to 56°, and we have followed its disappearance during the clotting of plasma in the course of a few minutes. Earlier authors have found it to be much more stable. The nature of this discordance is not yet clear, but it may be that the stability of the coinhibitor varies in a high degree from species to species. Other investigators have most often worked with dog and cat plasma, but we have found about the same lability of the substance in rabbit and human plasma as in ox plasma. The question is being investigated further.

In judging our results it must be taken into consideration that our method does not give quite quantitative results, especially when using ox plasma. It seems possible, however, from our semi-quantitative experimental results with certainty to make the following statements:

1. From the solution resulting from half saturation of ox plasma with ammonium sulphate it is possible to precipitate a less soluble component B, which contains practically no antithrombic material. The more soluble component C acts as an antithrombin.

2. By addition of heparin to component B is formed a powerful antithrombin, which is called *thrombin inhibitor*. B itself is called *thrombin coinhibitor*. Component C is activated only to a very slight extent by the addition of heparin.

3. After heating B to 56° for 5 minutes it cannot afterwards be activated by addition of heparin. Component C loses only about 10—20 per cent of its antithrombic activity by this treatment. At 40° C is stable, while B slowly loses its ability to be activated by heparin. B with heparin added is more stable against heating than B alone, but not in the same degree as C alone.

4. The thrombin inhibitor formed from B by addition of heparin seems to be a dissociable compound. This seems also to be the case with the inactive compound of thrombin and thrombin inhibitor (see equations  $A_1$  and  $A_2$ ).

5. The compound, called metathrombin, resulting from thrombin and the normal antithrombin, does not seem to dissociate to any measurable extent (see equation  $B$ ).

6. The compound B is only found in plasma, and is destroyed during the clotting. C is stable in serum, and found here in almost the same amount as in plasma.

7. B activated by heparin, *i. e.* thrombin inhibitor, neutralises thrombin much more rapidly than does C, the normal antithrombin.

From these results it seems clear that thrombin in plasma can be inactivated by two independent reactions, namely by a slow process due to the normal antithrombin, component C, and by a more rapid process due to the thrombin inhibitor formed from component B and heparin.

As the normal antithrombin, C is not activated by heparin, and as B, while in itself inactive, is activated by heparin, it follows that the component which may be activated by heparin, is not identical with the normal antithrombin, as is assumed especially by QUICK (1938). This is further suggested by the fact that, while the content of antithrombin in plasma is about the same as in serum, only plasma is increased in antithrombic power by addition of heparin.

That the normal antithrombin is not identical with the thrombin inhibitor resulting from heparin follows from the different dissociability of these compounds, the difference in stability on heating, and from the different velocity of their reactions with thrombin.

The views set forth here on the question of antithrombin and its relation to heparin have later been confirmed by VOLKERT (1941 and unpublished experiments) in experiments concerning the physiology of antithrombin, and with employment of quite different methods.

There remains the question, why our experiments on the properties of the thrombin coinhibitor are not in concordance with those of earlier authors. As already mentioned it is not easy to give a satisfactory answer to this, and the problem has to await further investigations, which are now being made, before it can be settled.



Nor do the experiments of other authors as reported in the literature give any obvious explanation of this question.

One of the possibilities is that the thrombin coinhibitor in plasma from different species may show different properties. According to HOWELL and HOLT (1918) the antithrombin content of cat plasma, which has been heated to 54°, is increased by the addition of heparin, and the same applies to cat serum. It should be remembered, however, that HOWELL's method for determination of antithrombin is rather inaccurate (cf. ASTRUP and DARLING (1942 b)). HOWELL and HOLT further found that the thrombin coinhibitor is *more stable than the normal antithrombin*, which is not increased in potency by the addition of heparin. They come to the conclusion that the factor which acts in conjunction with heparin in forming an antithrombin is a new substance which they call *pro-antithrombin*. The investigations were extended in subsequent papers (HOWELL (1925, 1926, 1928, 1935)). The substance is partially precipitated by acidification or by half saturation with ammonium sulphate and is assumed to belong to the euglobulines.

Since the original experiments of HOWELL the question of the mechanism of heparin in its action in the blood clotting process has been the subject of conflicting opinions. According to HOWELL (l. c.) heparin acts both directly as an *antiprotease* and by forming from an unknown precursor, called by him *pro-antithrombin* and by us *thrombin coinhibitor*, a real *antithrombin*, by us called *thrombin inhibitor*. The question of its rôle as an antiprotease will not be discussed here, as recent investigations show that here also a precursor is necessary for the inhibition (BRINKHOUS, SMITH, WARNER and SEEGER (1939), ASTRUP (1939)). Heparin obtained a prominent rôle in HOWELL's theory of blood clotting (cf. HOWELL (1935)). This question, however, concerns the mechanism of the formation of thrombin in a blood sample undergoing coagulation and falls outside the scope of this paper, which only deals with the formation of an antithrombic substance from heparin.

It is only in recent years that the work of HOWELL on this problem has been taken up by other authors. J. MELLANBY (1935) thus denies the existence of an additional substance and finds that heparin acts in itself as an antithrombin, but that sodium chloride is necessary for this action. He uses fibrinogen prepared by precipitating once with ammonium sulphate. HOWELL (1935)

disputes this finding, as he has shown that the substance in question is destroyed by heating; furthermore, he has used fibrinogen prepared by sodium chloride precipitation. MELLANBY finds that the action of heparin as an antithrombin is neutralised by thrombokinase, while the normal antithrombin is not influenced by this addition. Probably MELLANBY has worked with fibrinogen containing some thrombin coinhibitor.

SCHMITZ and KÜHL (1935) assume with MELLANBY that heparin acts as an antithrombin and that the results of HOWELL are due to inactivation of heparin in serum, as they find that serum inactivates heparin after incubation for some hours. Through this they will explain the instability observed by HOWELL. They fail, however, to explain why heparin cannot inhibit the clotting of purified fibrinogen.

QUICK (1936 a), in accordance with MELLANBY, finds that heparin is almost inactive in dialysed plasma and purified fibrinogen solution, and that the presence of sodium chloride and of HOWELL's co-factor is necessary for its action. In this way he brings MELLANBY's results into agreement with HOWELL's. It seems easier to remove the coinhibitor by precipitation with sodium chloride than with ammonium sulphate. He does not find that thrombokinase neutralises the action of heparin on thrombin. The necessity of the presence of HOWELL's co-factor for the formation of antithrombin was confirmed in later papers (QUICK (1937, 1938)). Especially the investigations set forth in the last-mentioned paper are of great importance, since it is found that both the normal antithrombin and the thrombin coinhibitor belong to the albumin fraction of the blood proteins. In contrast to our investigations he finds the co-factor present in plasma (rabbit) which has been clotted by means of thrombin. It is destroyed at 67°C, but he has not tried lower temperatures. He therefore assumes that heparin converts the normal antithrombin from a relatively weak thrombin-binding substance to a powerful inhibitor, while we, in accordance with the original conception of HOWELL, come to the conclusion that the normal antithrombin and the precursor for heparin-antithrombin are two different substances.

FERGUSON (1937) was first of the opinion that heparin acts directly as an antithrombin; later he confirms QUICK's findings of the activation of the serum albumin fraction of plasma by heparin (FERGUSON (1939, 1940)). He finds that the inhibitory action of crude and crystallised serum albumin itself is only very

small and is not increased by incubation, which is not in accordance with the results of most other authors, including ourselves. Heparin increases the action of crude but not that of crystallised albumin.

JAQUES and MUSTARD (1940) do not find the action of sodium chloride in accordance with that found by MELLANBY. They find the coinhibitor in the albumin fraction, but not in crystallised albumin, and they are here in agreement with FERGUSON. They use an unsatisfactory method for the determination of antithrombic action.

ZIFF and CHARGAFF (1940 a, b) fractionate albumin, but their results does not seem to be in accordance with ours.

As is pointed out by SMITH, WARNER and BRINKHOUS (1935) there must be distinguished between the amount of antithrombin present and the total amount which can be formed by addition of heparin. The total amount can not be increased by injection of peptone. These results have been confirmed recently by VOLKERT in unpublished experiments. India ink acts in the same manner (WARNER, BRINKHOUS and SMITH (1936)). The antithrombin present in peptone plasma was already in earlier times recognized as different from the normal antithrombin of plasma (MORAWITZ (1905)), and is now known to be due to the presence of heparin, (HOWELL (1925), QUICK (1935 b), WILANDER (1938)). Also the anticoagulant found in anaphylactic shock seems to be due to heparin (EAGLE, JOHNSTON and RAVDIN (1937), WATERS, MARKOWITZ and JAQUES (1938)).

Since the investigations of FISCHER (1935) it is known that heparin combines with proteins on the acid side of their isoelectric points. As thrombin itself is a very acid substance with isoelectric point about pH 4.4 (ASTRUP and DARLING (1941), SEEGER (1940)), it seems reasonable that an additional component is necessary for a combination between thrombin and heparin. Probably it may be a basic protein.

In this connections it is of interest that heparin also is an inhibitor for other enzymic reactions, thus for fumarase (FISCHER and HERRMANN (1937)) and for trypsin (HORWITT (1940), GLAZKO and FERGUSON (1940)).

From all these investigations by various authors it is seen that only on few points are the findings in harmony, and in this respect our investigations make no exception. It remains for future investigators to reconcile these different views.

### Summary.

1. The formation of an antithrombic substance from heparin is investigated.

2. In accordance with earlier authors the presence of a thermolabile substance in blood plasma is found to be necessary for the formation of an antithrombic substance from heparin. This substance is called *thrombin coinhibitor*.

3. The thrombin coinhibitor is found to be a more labile substance than hitherto assumed. It is destroyed after heating to 56° for 5 minutes. It is only found in plasma, and is not present in serum, while it is destroyed during the clotting process.

4. It is found that the thrombin coinhibitor is not identical with the normal antithrombin of plasma and serum.

5. To distinguish between the normal antithrombin and the antithrombic substance formed from heparin the last mentioned substance is called *thrombin inhibitor*, as the two substances are not identical.

6. Thrombin inhibitor is much more stable than the thrombin coinhibitor, and almost as stable as the normal antithrombin.

7. While normal antithrombin seems to combine with thrombin to form an undissociable compound, the compound between thrombin inhibitor and thrombin, as well as the compound between heparin and thrombin coinhibitor, seems to be highly dissociable.

8. Thrombin is inactivated much more rapidly by thrombin inhibitor than by antithrombin.

9. The discrepancies between our results and those of earlier authors are discussed.

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## The Distribution of the Blood between the Lower Extremities and the Rest of the Body.

By

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Quantitative data on the distribution of blood between the periphery and the central vessels are scarce, although the significance of the peripheral capacity for the regulation of the circulation has been much emphasized in earlier and recent works (c. f. ASMUSSEN, CHRISTENSEN and NIELSEN (1939), SILFVERSKIÖLD (1938) a. o.). SILFVERSKIÖLD (1938) has given an extensive review of the works on this problem up to 1938 and has contributed to our knowledge with a series of determinations on mice and guinea pigs. Very few determinations, however, have been made on human subjects and most of them are made by means of the plethysmograph on upper or lower extremities. (GRILL (1933) a. o.) Such determinations give very valuable informations concerning *changes* which might occur in the blood volume of the extremities, but do not give any hint of the total amount of blood in the limb. It is the purpose of this paper to present determinations of the amount of blood present in a well defined vascular area — i. e. the lower extremities of man — under different physiological conditions.

All determinations were made on two normal, male subjects, E. A. (34 years, 173 cm., 70 kg.) and R. J. (24 years, 172 cm., 60 kg.). Both have well developed musculature, E. A. an average layer of subcutaneous fat whereas R. J. is very lean. The experiments were made in the morning. The subjects rested for half an hour in the horizontal position

before the actual experiment but were not fasting. When the subject had been exposed to the conditions of the experiment for the appropriate time, cuffs, which had been previously placed proximally around the subject's thighs, were inflated to a pressure of 260 mm Hg by means of a cylinder containing compressed air. The volume of the blood in the part of the body proximally to the cuffs was now determined by the CO-method as previously described (ASMUSSEN (1941)) after which the pressure in the cuffs was released. The subject continued breathing from the metabolism apparatus containing the CO, and after about 10 more minutes a second blood-sample was drawn, from which the total blood volume was estimated. The difference between the last and the first blood volumes gives the volume of the blood in the legs under the experimental conditions.

The analyses of the CO-content of the blood were made on the Van Slyke apparatus according to VAN SLYKE and NEILL (1924).

As possible sources of error must be mentioned differences in the placing of the cuffs on the legs, and the possibility of developing stasis during the inflation of the cuffs. As the cuffs were always placed as proximally on the legs as possible, and as the inflating of the cuffs could be completed in a few seconds these errors must be negligible.

As the determinations were made over a period of several months, rather considerable variations in the blood volumes occurred. In some instances a very large fall in the total blood volume (e. g. in R. J. from about 4.8 l to 4.0 l, in E. A. from 5.7 l to 4.8 l) was found to coincide with periods of ailment. Such results were discarded. The smaller day-to-day variations exceeding the analytical accuracy of about 5 pCt could not be explained by known facts and presumably must be looked upon as normal physiological variations.

The conditions investigated were: rest in the horizontal position, and in a head-up and a head-down position (called hereafter the  $+60^\circ$  and the  $-60^\circ$  position, respectively, as the angle between support and the horizontal was of that magnitude); free standing; work on the bicycle-ergometer; and recovery from work, sitting on the bicycle with the feet supported.

For the experiments in the horizontal position the subject rested horizontally for 30—45 minutes, after which the cuffs were inflated and the rebreathing of the CO-mixture was started. The first blood sample was drawn after 10—15 minutes, upon which the pressure was released and 8—12 minutes more elapsed before the second blood sample was drawn.

The results are presented in table I.

The table shows, that in the two subjects 12—15 pCt of the total blood volume are present in the legs during rest in the horizontal position. Day-to-day determinations show rather large differences, especially in R. J. where the percentage blood volume of the legs varies between 11 and 21 % of the total blood volume. This might have been due to differences in the skin temperature

Table I.

Subject.		Lying horizontally			
		Blood vol. I (cuffs inflated) liters	Blood vol. II (total) liters	II—I (blood vol. of legs) liters	Blood vol. of legs in pCt of total
E. A. leg volume: 16 liters	mean . . . . .	5.02	5.73	0.71	12.4
	range . . . . .	4.86—5.25	5.46—6.03	0.60—0.80	11—14
	number of determinations .	8	8	8	8
R. J. leg volume: 11 liters	mean . . . . .	4.04	4.77	0.73	15.3
	range . . . . .	3.67—4.18	4.63—4.97	0.55—0.96	11—21
	number of determinations .	8	8	8	8

on the different days, but supplementary experiments in which the skin temperature was either lowered, by means of an electric fan blowing vigorously on the moistened legs of the subject, or increased, by means of warm blankets, showed no systematic differences. The differences are presumably due to the different activity of the subject previous to the experiments and indicate different states of vasodilatation in the muscles of his legs, persisting in spite of the resting period of at least half an hour. Although the absolute amount of blood in the legs is practically identical in E. A. and R. J., the relative amount is considerably larger in R. J., both in relation to the total blood volume and in relation to the total volume of the legs. This may be due to the well developed subcutaneous venous plexus of R. J., whose veins always appeared well filled under the skin.

The effects of tilting the subjects passively to head-up and head-down positions on the distribution of the blood have been demonstrated and discussed in earlier papers (ASMUSSEN, CHRISTENSEN and NIELSEN (1940)). It was shown by means of the plethysmograph, that a rather large shift of blood could appear on tilting and its effects on the general circulation were investigated. The present determinations confirm the earlier ones and give further the absolute volumes of the blood in the legs. After a 30 minutes rest in the horizontal position on the tilting table the subject was tilted either to the  $+ 60^\circ$  or to the  $- 60^\circ$  position. After 3—5 minutes in this position the main blood shift has taken



place (according to the plethysmograms of earlier works), and the cuffs were inflated. The subject now moved to an ordinary bed on which the blood volume determinations were carried out as usual. The results are shown in table II.

Table II.

Subject		+ 60°		— 60°	
		Blood vol. of legs liters	Blood vol. of legs in % of total bl. vol.	Blood vol. of legs liters	Blood vol. of legs in % of total bl. vol.
E. A.	mean . . . . .	1.27	22	0.71	13
	range . . . . .	1.12—1.51	19—26	0.59—0.80	11—14
	nbr. of det. . .	6	6	5	5
R. J.	mean . . . . .	0.86	19	0.51	11
	range . . . . .	0.82—0.90	18—19	0.40—0.57	9—12
	nbr. of det. . .	3	3	3	3

Table II shows, that the two subjects react differently to the tiltings. Whereas E. A. whose cutaneous veins in the horizontal position appeared empty, shows a marked increase in the blood volume of the legs on the tilting to + 60°, R. J., whose veins seemed well filled already in the horizontal position, exhibits a rather slight increase. On tilting to — 60° the opposite occurs. E. A. shows no changes in blood distribution compared to the horizontal, whereas R. J. shows a not inconsiderable decrease in blood volume of the legs. The fact that the blood volume of the legs in E. A. is unchanged after tilting to — 60° position, does not mean that nothing occurs in the vascular bed. The pale, almost yellow colour, which the skin of the legs exhibits during the period of anemia makes a strange contrast to the bluish-red colour of the legs when the cuffs are inflated in the horizontal position, and indicate, that the cutaneous venous plexus is nearly empty in the — 60° position. The unchanged capacity of the vascular bed of the legs then must mean, that other vessels dilate in the — 60° position, thus incidentally making up for the emptying of the cutaneous vessels. It seems natural to think of the arteries and capillaries in this connection as the lowered hydrostatic component of the arterial blood pressure makes a compensatory vasodilatation desirable for the adequate blood supply of the legs in the — 60° position.

The results indicate in agreement with earlier results (ASMUSSEN, CHRISTENSEN and NIELSEN (1939)) that, at least in some persons, a not insignificant amount of blood can be transferred from the legs to the central vascular area by tilting the subject. The possible practical consequences of this have been pointed out elsewhere. (ASMUSSEN, CHRISTENSEN and NIELSEN (1940)).

In order to get an impression of which importance the "venous pump" has on the shift of the blood which occur on changing position, a series of determinations were made, in which the subject, after the preliminary 30 minutes of resting in horizontal position, assumed a free standing position in which a natural "postural sway" was allowed. After 5 minutes of standing, the cuffs were inflated, and the blood volume determinations began. Table III shows the results.

Table III.

Subject		Free standing	
		Blood vol. of legs liters	Blood vol. of legs % of total blood vol.
E. A.	mean . . . . .	1.00	17
	range . . . . .	0.96—1.06	16—18
	nbr. of det. . .	4	4
R. J.	mean . . . . .	0.72	15
	range . . . . .	0.54—0.88	12—19
	nbr. of det. . .	4	4

The figures in table III indicate that the amount of blood present in the legs during free standing in both subjects is smaller than it was in the  $+60^\circ$  position. This can only be due to the pumping of the muscles during the postural sway and confirm the observations made by ASMUSSEN, CHRISTENSEN and NIELSEN (1939) a. o. that even small muscular contractions in the legs greatly influence the return flow of blood from the lower extremities. In R. J. the venous pump is able to keep the blood volume of the legs equal to what it was in the lying position, whereas it in E. A., although much smaller than in  $+60^\circ$  position, it still is well above the value in the horizontal position.

The effect of work with the legs on their blood filling is demonstrated by the figures of table IV. Two rates of work have been

investigated, viz. 410 mkg/min. and 820 mkg/min. The work was performed until a steady state was reached (10—15 min.) whereupon the cuffs were inflated during continued work. As soon as the pressure was on, the work was stopped, the subject placed on a bed, and the blood volumes determined.

It has been shown in an earlier paper (ASMUSSEN (1942)) that light work (360 mkg/min.) has no effect on the total blood volume as determined by the CO method. In the present experiments with somewhat heavier work the total blood volume also was found to lie within the range of the resting values, but it must be remembered, that the work was of rather short duration (10—15 min.) and that the actual determinations were made *after* work. Heavy work of longer duration is no doubt accompanied by a concentration of the blood (loss of water).

Table IV.

Subject		410 mkg/min.		820 mkg/min.	
		Blood vol. of legs liters	Blood vol. of legs % of total	Blood vol. of legs liters	Blood vol. of legs % of total
E. A.	mean . . . . .	0.87	17	0.60	11
	range . . . . .	0.70—1.15	13—21	0.53—0.71	9—11
	nbr. of det. . .	3	3	4	4
R. J.	mean . . . . .	0.74	17	0.83	18
	range . . . . .	0.71—0.76	15—18	0.69—0.96	14—21
	nbr. of det. . .	2	2	2	2

It is interesting to note, that although the work must cause a considerable vasodilatation in the working muscles — their metabolism is increased about 25, and 40 times respectively during the two rates of work (see ASMUSSEN, CHRISTENSEN and NIELSEN, (1939)) — the amount of blood present in the legs is but slightly increased, in the heavy work in E. A. even decreased. This means presumably, that the doubtless increased arterial and capillary capacity is counterbalanced or even overruled by the concomitant emptying of the veins called forth by the vigorous muscular movements (compare the plethysmographic results of GRILL (1933)). The great importance of the venous pump for the regulation of the circulation during the work is thus easily understood as a way by means of which the blood-shift from the peripheral veins to the central veins, heart and arteries is accomplished.

The veins of the muscles no doubt serve as a blood reservoir, which automatically is emptied when work is commenced.

If the work is discontinued, the pumping action of the muscles ceases and the veins will be filled quickly to capacity. This in connection with the still enlarged capillary bed will demand a large volume of blood, thus endangering the general circulatory regulation (comp. MATEEFF (1935)). The amount of blood pooling in the legs immediately after work was determined on the two subjects, the cuffs being inflated 20 to 30 seconds after cessation of work. Two rates of work were tested and the results came out as shown in table V.

Table V.

Subjects		After 820 mkg/min.		After 1,230 mkg/min.	
		Blood vol. of legs liters	Blood vol. of legs % of total	Blood vol of legs liters	Blood vol. of legs % of total
E. A.	mean . . . . .	1.47	26	1.34	24
	range . . . . .	1.32—1.62	25—27	1.16—1.53	22—26
	nbr. of det. . .	2	2	2	2
R. J.	mean . . . . .	1.28	23	1.19	25
	range . . . . .	1.02—1.40	19—26	0.97—1.46	19—30
	nbr. of det. . .	3	3	3	3

It will be seen, that under these circumstances up to 26 pCt of the total blood volume can be accumulated in the legs distal to the cuffs. This means a "loss" of blood from the rest of the body of about 15 pCt of the total blood volume and comes close to the amount of blood that can be lost without causing troubles to the organism. In addition it may be mentioned, that the muscles that could be cut off from the circulation in these experiments, only represent about 40 pct. of the working muscles, so that the actual displacement of blood must be assumed to be still larger.

### Summary.

The amount of blood present in the legs under different conditions of work and rest is determined by means of the CO-method on two subjects. During rest in the horizontal position it was 12 and 15 pCt of the total blood volume. On tilting the subjects 60° to a head-up position it increased to 22 and 19 pCt, and on

tilting the subjects  $60^\circ$  to a head-down position it was 13 and 11 pCt, respectively. In free standing it was 17 and 15 pCt of the total blood volume. During work (410 and 820 mkg/min.) it was but slightly changed (17 and 11 pCt in one subject, 17 and 18 the other subject) whereas after work it was markedly increased in both subjects (to 26 pCt and 25 pCt).

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## Investigations on the Electrolyte Content of Gastric Juice.

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Among the very numerous investigations dealing with the gastric secretion only relatively few have had for their object a comprehensive account of the composition of the gastric juice. Approximately complete analyses of the inorganic constituents are thus quite rare, and the first important contributions of this kind (SCHOUHOWA-SIMANOWSKY (1894), ROSEMAN (1907)) are still frequently cited in the literature.

The treatment of the subject is actually even less exhaustive than one would imagine from the small number of papers published. This is especially due to the circumstance that the gastric juice cannot be conceived as a physiological product of a definite composition, like for example milk; on the contrary, it presents significant differences under different conditions. To all appearances the gastric juice is a mixture of the secretion of several dissimilar glandular elements, and its variable character must undoubtedly be regarded — at least partly — as a consequence of independent changes of the activities of the individual cell types. Perhaps it is even necessary to assume a varying composition of the secretion from cells of the same type.

Because of these and other similar facts it is usually impossible, without reservation, to compare analytical results which are not obtained under identical experimental conditions.

The evaluation of the data becomes particularly difficult when the investigations do not apply to the same regions of the stomach.

For example, findings which are based on experiments with animals operated upon according to the methods of HEIDENHAIN and PAVLOV cannot be compared directly with data referring to the secretion of the stomach as a whole.

Though we still lack an understanding of the true nature of the processes which result in the variations of the composition of the gastric juice, we know from a series of examples that the relative proportion of certain single constituents may exhibit well defined variations under varying external conditions.

Thus, according to BABKIN (1930—31) and other investigators, gastric juice which is secreted after histamine injection is much poorer in pepsin than "hunger juice", for example, and both kinds contain less mucus than a secretion produced by means of pilocarpine.

In the same connection we may also mention the changes of the hydrochloric acid concentration which appear, *inter alia*, when the rate of secretion is changed.

It is generally assumed (PAVLOV (1898), HOLLANDER and COWGILL (1931), TEORELL (1933)) that the acidity gradually rises with increasing rate of secretion, and, conversely, falls when the amount of gastric juice secreted per unit of time diminishes.

A reduction of the hydrochloric acid content may also arise when the gastric juice remains in contact with the gastric mucosa for a longer period of time (GAMBLE and McIVER (1928)).

Many theories have been advanced in explanation of the observed variations in the acidity of the gastric juice.

PAVLOV (1898) was of the opinion that the secretion of the acid producing cells is of constant strength, and that the variation of the acidity which may be observed when investigating the gastric juice is due to the influence of neutralizing mucus.

ROSEMANN (1921), on the other hand, maintained that the acid producing cells themselves are capable of secreting hydrochloric acid of varying concentration, dependent on the nature and intensity of the stimulus.

ROTH and STRAUSS (1899), MACLEAN and GRIFFITHS (1928), APPERLY and NORRIS (1930) and others emphasize the idea of a "Verdünnungssekretion" as a link in the acid regulating mechanism of the stomach. In general, these authors find a decrease with time of the acidity of the alimentary gastric juice, and assume that the falling hydrochloric acid concentration finds its explanation in the admixture of an aqueous salt-containing secretion —

according to MACLEAN and GRIFFITHS of an approximately 0.11 N solution of neutral chloride.

The regurgitation theory, especially supported by BOLDYREFF (1908) was long a subject of lively discussion; the idea was that the regurgitation of alkaline intestinal contents from the duodenum into the stomach produces the fluctuations in the acidity of the gastric contents. Of course, the theory may be ignored in experiments where all connection with the intestine is broken off.

It is considerably more difficult to test hypotheses like that of HOLLANDER (1932), which presupposes, side by side with the production of hydrochloric acid, the secretion of a neutralizing fluid containing bicarbonate. Yet, lowering of the acidity, produced in this way, must necessarily lead to an increased evolution of carbon dioxide in the cavity of the stomach, and it should be possible in consequence to register the extent of the neutralization.

TEORELL (1933) was unable to verify any such production of carbon dioxide, and was therefore inclined to reject the theory. Instead he strongly advocates the idea of considering the acidity regulation a diffusion process. The hydrochloric acid is secreted at a high concentration, and any lowering of the acidity observed is brought about by an exchange of hydrogen ions with alkalis through the stomach mucosa.

In planning the investigations of which the following is a preliminary report, it was the intention (1) to prepare a general account of the composition of the gastric juice under certain conditions, and (2) to contribute to the understanding of the so-called acidity regulation.

Out of regard to the formulation of the problem, and in order to simplify the interpretation of the results, it was chosen to confine the investigation to that region of the stomach in which the hydrochloric acid is produced, i. e., the fundus.

Rather than arranging tedious and complicated experiments by means of fistula- or pouch-operations, it was preferred to sacrifice a fresh animal on each experiment which therefore had to be carried out under narcosis and completed in one session. Cats were used.

It has been endeavoured to make the experimental conditions as simple as possible; hence the changes in the composition of the gastric juice which are desired are produced solely by means of an arbitrary change in the amount of secretion per unit time.

It should be added, however, that the gastric juice in some of



the experiments is secured at certain intervals,  $\frac{1}{4}$  or  $\frac{1}{2}$  hour, while in others a continuous aspiration has been carried into effect so that the gastric juice is withdrawn from the stomach immediately after the secretion.

In all experiments the secretion is produced by means of histamine. The doses are adjusted empirically, according to the intensity of secretion desired.

7 experiments are mentioned. In 5 of these the gastric juice is collected in 2 fractions — one during stronger, the other during weaker secretion.

### Methods.

The cats weighed from 2.5 to 3.2 kg and were apparently healthy and well fed.

Chloralose and urethane were used for the narcosis, 0.05 and 0.5 g per kg body weight respectively.

Histamine hydrochloride was used in a 0.1 % solution, injected subcutaneously in doses of 0.25—0.75 mg.

Prior to the first experiments the animals had been starved for 24 hours, but since it appeared that this procedure affected the secretory capacity too much, it was tried in the last two experiments to withhold solid food from the cat during the last 24 hours while feeding it milk until about 10 hours before the beginning of the narcosis.

Through a cut in the center line of the abdominal wall a rubber-coated clamp is placed on the transition between the fundic and the pyloric regions so that any connection between these two regions becomes impossible. Next, a double tube is lowered through the pharynx and the esophagus, the tube consisting of a rigid, laquered catheter with a tight fitting soft Nélaton catheter inside.

The outer tube is introduced only so far that the end, which is equipped with a special rubber cuff, just lies in the cardia.

2 catgut ligatures are tied around the lowest part of the esophagus wall which in this way fits closely to the rubber cuff lying inside.

The Nélaton catheter is longer than the outer tube and extends all the way into the stomach, with a 6—7 cm long, free piece which is perforated in several places. In this way it is possible, by means of a calibrated glass syringe, to withdraw the gastric juice collected in the isolated fundus cavity.

Care is taken to prevent evaporation and admixture of foreign matter.

The hydrochloric acid is determined by titration with a 0.1 N  $\text{CO}_2$ -free solution of sodium hydroxide.

It is preferred to titrate to  $\text{pH} = 5$ , on the basis of the assumption that the isoelectric point of the mucins, etc., in gastric juice, as in the case of most proteins, lies in the proximity of this value.

In this way it is assured, as far as possible, that the amount of NaOH consumed is exclusively a measure of the HCl-content of the gastric

juice. Brom cresol green is used as an indicator; the desired end-point of the titration is found by comparison with the colour of a citrate solution (according to SØRENSEN (1910)), to which is added the same amount of indicator as in the samples of the gastric juice.

The difference between double analyses but rarely exceeds 0.5 %.

In principle chloride is determined according to the method of WILSON and BALL (1928) the silver chloride precipitate, however, being filtered before the titration. In this way it is possible to obtain reliable and accurate results. The error of the chloride determination is 0.3 % at the most.

The sodium analyses are made by means of a micro-apparatus according to the method of BARBER and KOLTHOFF (1928). With the available amounts of material the error will usually be less than 1 %; the same is true of the potassium determinations which, in the main, are made according to the procedure described by PETERS and VAN SLYKE (1932), reduced to microscale.

Ammonium is measured in the "units" proposed by CONWAY and BYRNE (1935), but the directions of the authors have not been followed in all details. Thus the titration is carried out with an approximately N/70 sodium hydroxide solution, using brom cresol purple as indicator; likewise, it is not found necessary to shorten the distillation period when the material is gastric juice. If care is used, the results are reliable; the error is about 2 % at the most.

Calcium and magnesium are determined using a technique which closely follows the micro-procedure proposed by BENEDETTI-PICHLER (1924) and BENEDETTI-PICHLER and SCHNEIDER (from HECHT and DONAU (1940)) respectively.

Phosphates are determined according to FISKE and SUBBAROW (1925).

The total nitrogen of the gastric juice is determined by combining the Kjeldahl digestion proposed by A. C. ANDERSEN and NORMAN JENSEN (1931) with distillation in the apparatus of PARNAS and WAGNER (1921).

The pepsin values are found by means of the method of BENT ANDERSEN (1938). The standard curve is prepared from analogous experiments with solutions containing known amounts of Merck Pepsin.

Hydrochloric acid, total chloride, ammonium, total nitrogen and pepsin are determined directly in separate 1 ml samples of the gastric juice.

For the determination of sodium, potassium, calcium, magnesium and phosphorus, some 15 ml of gastric juice are pipetted off and evaporated in a quartz dish, concentrated sulphuric acid is added, the solution evaporated, and the residue ignited.

After heating with dilute hydrochloric acid for some time the total ignition residue is dissolved and then transferred quantitatively to a measuring flask and made up to 50 ml by means of distilled water.

15 ml are withdrawn from this flask for the sodium and potassium analyses. Calcium and phosphates are removed in a suitable manner, whereupon follows treatment with concentrated sulphuric acid, ignition,

etc. Finally, the alkali metal sulphates are transferred to a weighing glass; after evaporation in a drying oven the salts are weighed and then dissolved in 1 ml of water. The samples for the determination of sodium and potassium are obtained from this solution by means of micro-pipettes.

With only a few exceptions, each result is the average of double determinations.

## Results and Discussion.

The results of the determinations of the acid and total chloride are recorded in table 1.

Experiments XII, XV, XVII, XVIII and XX show plainly the well known connection between acidity and rate of secretion. The concentration of hydrochloric acid in the samples analysed varies from 137.3 to 167.0 milli-equiv./liter, i. e., within an interval of 29.7 milli-equiv./liter.

There may be reason to emphasize that the acid values on the average are quite high in comparison with the observations of most authors. Even at a secretion as reasonably small as 1.14 ml per 15 minutes we find 139.45 milli-equiv./liter. Concentrations like the highest ones in this material (167 milli-equiv./liter) are found but rarely in the literature.

The same may be said with regard to the chloride values. The difference between the highest chloride concentration, 190.1, and the lowest, 167.6 milli-equiv./liter, is 22.5 milli-equiv./liter, thus nearly the same as the maximal amplitude in case of the acid figures.

It will be seen from the figures in table 1 that the total chloride also shows a regular connection with the rate of secretion. In analogy with the changes in the acidity, the chloride concentration rises and falls with the amount of gastric juice secreted.

The individual experiments in which the gastric juice is collected in two fractions indicate, moreover, a certain parallelism between the concentrations of hydrochloric acid and total chloride, insofar as they are displaced about equally in the same direction when the rate of secretion is changed. The discrepancies seem to be somewhat larger, however, in case of the hydrochloric acid, so that the difference between the chloride and the hydrochloric acid figures is the smallest in the proximity of the maximal values.

These results are completely in line with those of HOLLANDER (1931—32) and TEORELL (1933).

Table 1.

Secretion in ml per  $\frac{1}{4}$  hour.

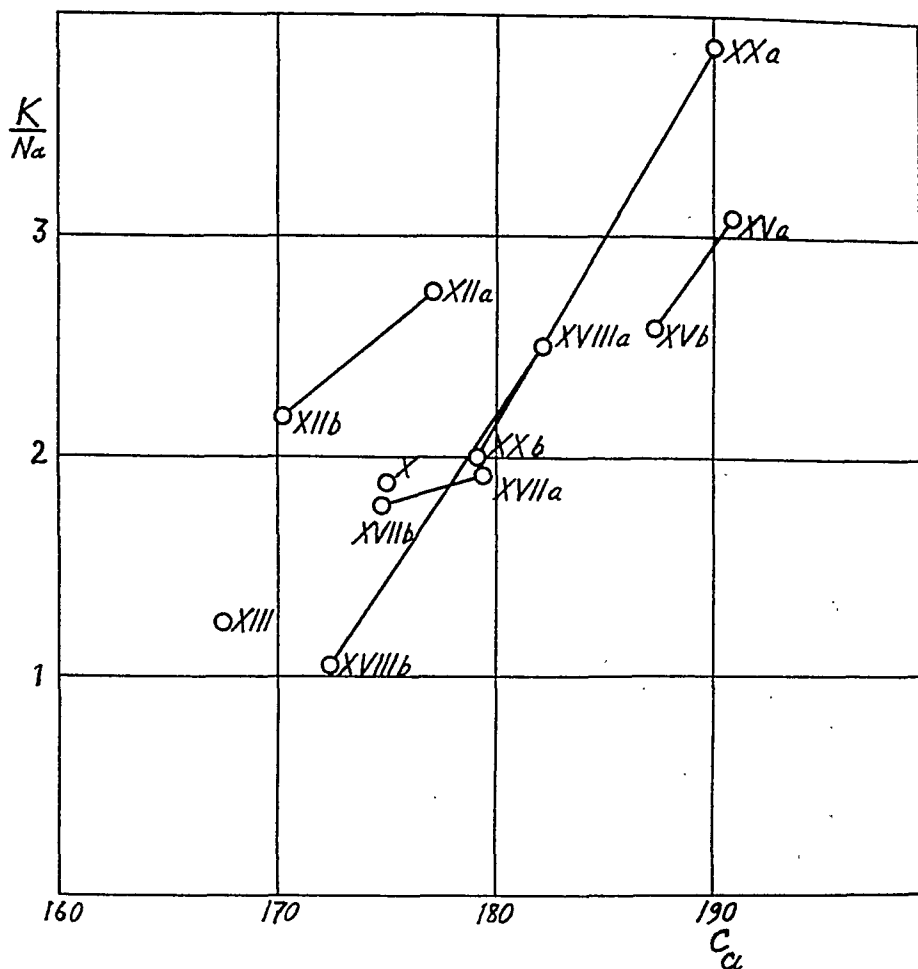
Concentration of Cl and HCl expressed in milli-equivalents per liter of gastric juice.

	X Aspira- tion at $\frac{1}{4}$ -hour intervals	XII a Aspira- tion at $\frac{1}{4}$ -hour intervals	XII b Aspira- tion at $\frac{1}{4}$ -hour intervals	XIII Aspira- tion at $\frac{1}{4}$ -hour intervals	XV a Aspira- tion at $\frac{1}{4}$ -hour intervals	XV b Aspira- tion at $\frac{1}{4}$ -hour intervals	XVII a Aspira- tion con- tinu- ously	XVII b Aspira- tion at $\frac{1}{2}$ -hour intervals	XVIII a Aspira- tion con- tinu- ously	XVIII b Aspira- tion con- tinu- ously	XX a Aspira- tion con- tinu- ously	XX b Aspira- tion con- tinu- ously
Secretion .....	2.5	3.64	1.87	1.5	4.38	2.19	2.17	1.23	5.29	1.14	5.25	1.66
Cl .....	175.0	177.0	170.3	167.6	190.9	187.28	179.4	174.8	182.14	172.43	190.1	179.2
HCl .....	152.0	152.5	137.3	137.3	163.15	155.2	158.3	153.4	162.9	139.45	167.0	151.9
Cl-HCl .....	23.0	24.5	33.0	30.3	27.75	32.08	21.1	21.4	19.24	32.98	23.1	27.3
Total Base .....			31.99	29.70		30.75		20.71	17.47	31.92	22.15	26.46

Table 2.

Secretion in ml per  $\frac{1}{4}$  hour.Concentration of Cl, HCl, Na, K,  $\text{NH}_4$ , Ca, Mg expressed in milli-equivalents per liter of gastric juice.

	2.5	3.64	1.87	1.5	4.38	2.19	2.17	1.23	5.29	1.14	5.25	1.66
Secretion .....	175.0	177.1	170.3	167.6	190.9	187.28	179.4	174.8	182.14	172.43	190.1	179.2
Cl .....	152.0	152.5	137.3	137.3	163.15	155.2	158.3	153.4	162.9	139.45	167.0	151.9
Na .....	5.65	5.25	8.65	11.12	4.26	5.71	5.45	5.80	4.47	13.15	3.85	7.51
K .....	10.60	14.45	18.78	13.76	13.09	14.72	10.88	10.23	11.18	13.75	14.89	15.04
$\text{NH}_4$ .....	—	3.30	2.30	3.27	8.97	8.90	4.34	3.27	1.53	4.20	3.13	3.54
Ca .....	1.86	—	2.26	1.55	—	1.42	—	1.41	0.29	0.82	0.28	0.97
Mg .....	—	—	0	0	—	0	—	0	0	0	0	0
Sum of Cations .....	—	—	169.29	167.0	—	185.95	173.47	174.11	180.37	171.37	189.15	178.36
Na+K .....	16.25	19.70	27.43	24.88	17.35	20.43	15.83	16.03	15.65	26.90	18.74	22.55
K/Na .....	1.88	2.75	2.18	1.24	3.08	2.58	1.91	1.77	2.50	1.05	3.86	3.00



The largest and smallest difference between corresponding chloride and hydrochloric acid values is 33.0 and 19.2 milli-equiv./liter respectively, the mean difference being 26.3 milli-equiv./liter.

These quantities correspond very closely to the contents of neutral chloride, i. e., cations other than  $H^+$  or total base in the samples, as evidenced by all the more complete analyses (see table 1).

Although it thus seems to be true that the amount of neutral salts per unit of volume of gastric juice decreases with increasing concentration of hydrochloric acid, these data nevertheless give no support to the contention of GILMAN and COWGILL (1932—32) and others, that the neutral chloride content is entirely insignificant at maximal rate of secretion and maximal acidity.

ROSEMAN (1907) demonstrated that there is more potassium than sodium in the "hunger juice"; the analyses of all the samples in the present investigation give the same result (table 2).

It may be added that the sodium is found to vary from 3.85 to 13.15 milli-equiv./liter, potassium from 10.23 to 18.78 milli-equiv./liter.

It is now found that there exists a distinct relationship between the potassium-sodium ratio and the concentration of total chloride, noticeable by comparison of different experiments (see fig.). It is seen moreover, in the experiments where two portions of gastric juice are analysed, that a lowering of the concentrations of both sodium and potassium occurs during more ample secretion, though sodium varies considerably more than potassium within each individual experiment.

The ammonium content is rather fluctuating: 1.53 to 8.97 milli-equiv./liter, but does not appear to have definite connection with the other inorganic compounds. Continued investigations of this very point are planned.

Calcium is found in only small amounts: from 0.28 to 2.26 milli-equiv./liter. The concentration is apparently higher at lower rates of secretion.

It has not been possible to demonstrate the presence of magnesium in any of the samples, not even microscopically.

Phosphorous is found only as traces (table 3).

Table 3.

P and N expressed in mg per 1 liter of gastric juice.

The pepsin figures expressing percentages of Merck Pepsin solution.

	X	XII b	XIII	XVII b	XVIII a	XVIII b	XX a	XX b
P .....	1.75	2.78	2.5	1.56	0	2.5	2.34	4.6
N .....					150	213	200	231
Pepsin .....					0.0108	0.0130	0.0113	0.0300

The pepsin content (table 3) is determined in two of the experiments (XVIII and XX), though only the result of XX can be considered reliable since the analyses in XVIII were carried out after the gastric juice had remained in the refrigerator for a longer period.

After subtraction of the ammonia nitrogen, the quantities of

total nitrogen are an expression of the total amount of mucin and other protein-like substances (table 3).

The figures are relatively small in comparison with most of the results of the literature.

Summing up, it may be said that the variations which are observed in the composition of the gastric juice to a very large extent depend on the rate of secretion, but it is hardly of any importance whether the aspiration of the gastric juice is carried out continuously or discontinuously at intervals of  $\frac{1}{4}$  to  $\frac{1}{2}$  hour.

In order to find out what conclusions regarding the acidity regulation we may draw from the data obtained, it is expedient first to investigate whether any of the existing theories can satisfactorily explain the present findings.

If we assume that the composition of the secretion of the acid producing cells is primarily constant, it is necessary first to consider the possibility that the varying acidity and salinity of the gastric juice are due to admixture of secretions from the other glandular elements of the stomach.

In all probability histamine acts as a specific stimulus for the hydrochloric acid producing glandular cells. Therefore, if the primary hydrochloric acid secretion has a certain, constant composition, this will be reproduced by the gastric juice with an approximation which becomes better the more the secretion increases in strength.

In that case the object will be to determine the composition of the secretion in question, but it is not possible to offer an exact solution of this problem on the basis of the available results. At the most, it may be possible in the experiments where the gastric juice is obtained in two fractions to estimate how we must suppose the primary hydrochloric acid secretion to be composed under the arbitrary assumption that the "Verdünnungssekret" also is of constant composition and moreover is secreted at a constant rate, independently of the rate of the hydrochloric acid secretion.

If  $A_q$ ,  $B_q$ ,  $M_q$  and  $M'_q$  are the concentrations of any electrolyte ( $q$ ) in the primary hydrochloric acid secretion, the "Verdünnungssekret", the "fast", and the "slow" fractions respectively, we obtain

$$\begin{aligned} x \cdot A_q + y \cdot B_q &= v \cdot M_q \quad (\text{"fast" secretion}) \\ x' \cdot A_q + y' \cdot B_q &= v' \cdot M'_q \quad (\text{"slow" secretion}) \end{aligned} \quad \dots \quad (1)$$

where  $x$ ,  $y$ ,  $v$ ,  $x'$ ,  $y'$ ,  $v'$ , are the rates of flow of the different secretions in the two cases.

Hence putting  $y = y'$   
 (i. e. the same rate of flow of the "Verdünnungssekret" during fast and slow secretion)  
 and

$$\begin{aligned}x + y &= v \\x' + y' &= v'\end{aligned}$$

we obtain

$$A_g = \frac{vM_g - v'M'_g}{v - v'} \dots \dots \dots (2)$$

The results are somewhat deviating, however, in the different experiments (see table 4), but it should nevertheless be safe to conclude from these data that the secretion from the hydrochloric acid producing cells is not a pure hydrochloric acid solution, but, at any rate, also contains discernible amounts of potassium chloride.

Table 4.

*Concentration of hypothetical hydrochloric acid secretion.*

Chloride .....	185 — 195	milli-equiv./liter.
Hydrochloric acid.....	169 — 174	" " "
Potassium .....	10.5— 22	" " "
Sodium .....	2.1— 2.2	" " "

The figures for hydrochloric acid are of the same order of magnitude as the values extrapolated by HOLLANDER (1932).

In one experiment (XX) it is also tried to make a calculation on the basis of the pepsin content of the two fractions, assuming that the variation of the electrolytes is due to an admixture of secretion from the pepsin producing cells in which case the pepsin content should probably be proportional to the electrolyte admixtures.

From equation (1) we find

$$A_g \left[ \left( 1 + \frac{x}{y} \right) - \left( 1 + \frac{x'}{y'} \right) \right] = M_g \left( \frac{x}{y} + 1 \right) - M'_g \left( \frac{x'}{y'} + 1 \right)$$

and if it is assumed that the "Verdünnungssekret" has a constant pepsin (and electrolyte) concentration we obtain

$$\frac{p'}{p} = \frac{y'}{x' + y'} \bigg/ \frac{y}{x + y} \dots \dots \dots (3)$$

where  $p'$  and  $p$  are the pepsin concentrations of the "slow" and "fast" fractions respectively.

Hence

$$A_g \cdot \frac{p' - p}{p'} = M_g - M'_g \cdot \frac{p}{p'} \dots \dots \dots (4)$$



The composition of the primary hydrochloric acid secretion as calculated from experiment XX is then as follows:

Chloride . . . . .	196.8	milli-equiv./liter
Hydrochloric acid . . . . .	176.0	» » »
Potassium . . . . .	14.8	» » »
Sodium . . . . .	1.6	» » »

It seems worth while to investigate whether it is at all possible that the changes of the composition of the gastric juice which we find at the transition from faster to slower secretion may conceivably be caused by the admixture of any secretion of "reasonable" composition (see below).

If we consider the "slow" fraction of the juice as a mixture of the rapid one and some "Verdünnungssekret" it is possible by simple calculation to establish how the contents of the different electrolytes in the "Verdünnungssekret" must change when we successively attribute an increasing series of chloride values to this secretion.

If  $z$  is the rate of flow of the "fast" fraction and  $n$  that of the "Verdünnungssekret", the other letters having the same significance as before, then

$$z \cdot M_q + n \cdot B_q = (z + n)M'_q$$

and hence for example

$$\frac{z}{n} M_{Cl} + B_{Cl} = \left( \frac{z}{n} + 1 \right) M'_{Cl}$$

or similarly

$$\frac{z}{n} M_{Na} + B_{Na} = \left( \frac{z}{n} + 1 \right) M'_{Na}$$

and

$$\frac{z}{n} M_K + B_K = \left( \frac{z}{n} + 1 \right) M'_K$$

Inserting different values for  $B_{Cl}$  the corresponding values of  $\frac{z}{n}$  may be obtained and from these again  $B_{Na}$ ,  $B_K$  etc. may be calculated.

By way of illustration an example of the calculation is given (XVIII):

$B_{Cl}$	$\frac{z}{n}$	$B_H$	$B_{HCO_3}$	$B_K$	$B_{Na}$
50	12.6		156	46.1	123.2
60	11.6		132	43.6	114.4
70	10.6		109	41.0	105.7
80	9.5		83	38.2	96.1
90	8.5		60	35.6	87.4
100	7.5		36	33.9	78.7
110	6.4		11	30.2	69.1
120	5.4	13		27.6	60.4
130	4.4	36		25.1	51.6
140	3.3	62		22.2	42.0

It is found in this way that at a chloride value of around 80 milli-equiv./liter the "Verdünnungssekret" changes from acid to alkaline reaction so that at lower chloride values the secretion is alkaline and contains an amount of bicarbonate which increases with decreasing chloride concentration, while at higher chloride values the secretion must be acid, and more so the higher the chloride value attributed to it. The volume of the secretion necessary to bring about the required change in electrolyte composition increases with increasing chloride value, its total concentration of electrolytes being minimum when  $\text{Cl} = 80$  and increasing to both sides of this point.

Since according to TEORELL (1933) it is unlikely that bicarbonate in larger amounts is the cause of the "neutralization" of the fast secretion, the hypothetical "Verdünnungssekret" must be neutral and have a total concentration of electrolytes of about 80 milli-equiv./liter, and since at the same time the calculation shows that the potassium content in this case must be between 17 and 30 we have evidently to deal with a strongly hypotonic secretion with a very high potassium concentration. The only analytical data in the literature with which these figures may be advantageously compared are the data for the pylorus secretion found by GAMBLE and McIVER (1928) and by TAKATA (1922—23), viz.,  $\text{Cl}$ : 158—150,  $\text{K}$ : 8.8—15 milli-equiv./liter, where the former figures (GAMBLE and McIVER) probably are the most reliable ones. This comparison shows that if the change of the composition of the "fast" secretion is caused by a secretory admixture, this admixture must be very different from the pylorus secretion, and in general be of a very unlikely composition.

The conclusion must be that the observed changes in the concentration of the electrolytes of the gastric juice, including that of the hydrochloric acid, cannot be explained solely as consequences of dilution, neutralization, or, on the whole, of an admixture of any secretion.

Nor does it seem possible to confirm TEORELL's diffusion theory on the basis of these results. In particular, it does not agree with the strange phenomenon that not only the sodium, but also the potassium concentration is increased in the fraction collected during slower secretion.

Of course, we must always bear in mind that the electrolyte fluctuations may possibly be an expression of a combined effect of several dissimilar processes; for example, diffusion and an ad-

mixture of secretion rich in potassium might be considered a plausible cause of the variations found.

Thus it seems possible to interpret the findings in question, even when assuming a constant composition of the primary hydrochloric acid secretion, but there are several facts to indicate that this assumption does not hold true, or, at any rate, is valid only within certain limits.

For example, small changes of the composition of the primary secretion, in combination with the admixture of a small amount of another secretion, possibly containing a little bicarbonate, would be sufficient to explain the electrolyte fluctuations observed.

On the other hand, the present relatively uniform data contain no direct proof of the assumption that large changes in the composition of the gastric juice should be produced by means of a mechanism which is in agreement with ROSEMANN'S assumption of a primarily varying hydrochloric acid secretion.

### Summary.

1. A brief presentation is given of different factors affecting the composition of the gastric juice.

2. The results of a series of analyses of gastric juice are reported, the gastric juice in question being secreted by the isolated fundus in cats under the influence of histamine. The investigations are focused on the changes in concentration of the electrolytes of the gastric juice, accompanying fluctuations of the rate of secretion.

3. The concentrations of hydrochloric acid and total chloride rise with increasing rate of secretion; the contents of neutral chloride diminish at the same time, without however falling much below 20 milli-equiv./liter.

4. The ratio between the potassium and the sodium concentrations is a function of the concentration of total chloride.

5. The concentrations of potassium and — especially — of sodium decrease when the rate of secretion increases.

6. With HEIDENHAIN-PAVLOV'S theory regarding the acidity regulation as a starting point, an attempt is made to estimate the composition of the primary hydrochloric acid secretion; in all probability this secretion contains a not insignificant amount of potassium chloride.

7. After a treatment of the data in the light of different theories, the conclusion is reached that it is hardly possible to maintain the thesis of the constancy of the primary hydrochloric acid secretion in its strictest sense.

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## A New Method for Direct Electric Registration of the Intra-Arterial Pressure in Man, with Examples of its Application.

By

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The convenient indirect method for measuring the arterial blood pressure is uncertain and leaves room for many errors. The development of a physiologically and clinically easy method for direct mensuration of the intra-arterial pressure will therefore be of value.

When measuring the pressure directly, one does not, as when measuring it indirectly, cause any change in the hydrodynamic condition in the vessels. With the indirect method, the compression introduces an increase of pressure due partly to reflection, partly to the transformation of the "kinetic" component into a pressure component, both of which enter into the measurement of the resulting pressure.

Further difficulties arise when it comes to estimating the diastolic pressure by the indirect method. Here the usually applicable criteria introduce a very considerable uncertainty, which under certain conditions, such as intense muscular effort, make measuring impossible. These uncertainties, together with the rigidity of the tissue and *particularly of the vessels themselves* and the inaccuracy that the pressure is not measured at a well-defined point of the vascular system, make the indirect method unsuitable for a quantitative estimation of the systolic, and

especially of the diastolic pressure.<sup>1</sup> Besides, the indirect method presupposes uniformity of the individual heart beats and permits neither registration of the changes in pressure within a given time nor continuous registration. A number of attempts have been made to devise a method for measuring the pressure in the arteries directly, by arterial puncture.<sup>2</sup> In the hands of an inexperienced operator this procedure may, of course, involve certain difficulties, but especially in America it has been used for many purposes, and with some practice it can be carried out without any more discomfort to the patient than a venipuncture. In their present form these methods are unfortunately, on account of the coagulation in the cannula, unsuitable for experiments lasting more than about fifteen minutes, unless special measures are taken.

The first manometers for *quantitative* mensuration of the intra-arterial pressure were constructed by FRANCK and described in his classical papers from 1911 and 1915. Later, the method was further developed by WIGGERS (1928) and by BROEMSER (1927) in the form of a glass plate manometer, which was used by WOLF and VON BONSDORFF (1931) for measuring the intra-arterial pressure in man. The drawback to the practical use of manometers of this type is the optical or mechanical transmission for visualisation of the movements of the glass membrane, because the slightest displacement of the manometer compromises the registration of the pressure curve. Electric transmission of the movements of the membrane would therefore offer great advantages.

The possibilities of effecting an electric transmission are numerous. Change of resistance as way of transmission was used by GRÜNBAUM (1897), GARTEN (1915) and SCHÜTZ (1931) in a pressure sound for measuring the intra-vascular pressure in animals. A manometer with photoelectric transmission was devised by REIN (1940). For measurements on man, the piezo-electric effect has been used by LANGEVIN and GOMEZ (1931), FABRE (1940) and MACLEOD and COHN (1941), but the method has not been adopted to any great extent, probably because only the variations in pressure, but not the mean pressure, can be followed, and because the more sensitive crystals are greatly influenced by temperature changes and show hysteresis.

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<sup>1</sup> A discussion of the oscillometry and its partly uncertain criteria lies outside the scope of this communication.

<sup>2</sup> For literature see VON RECKLINGHAUSEN's monograph (1940).

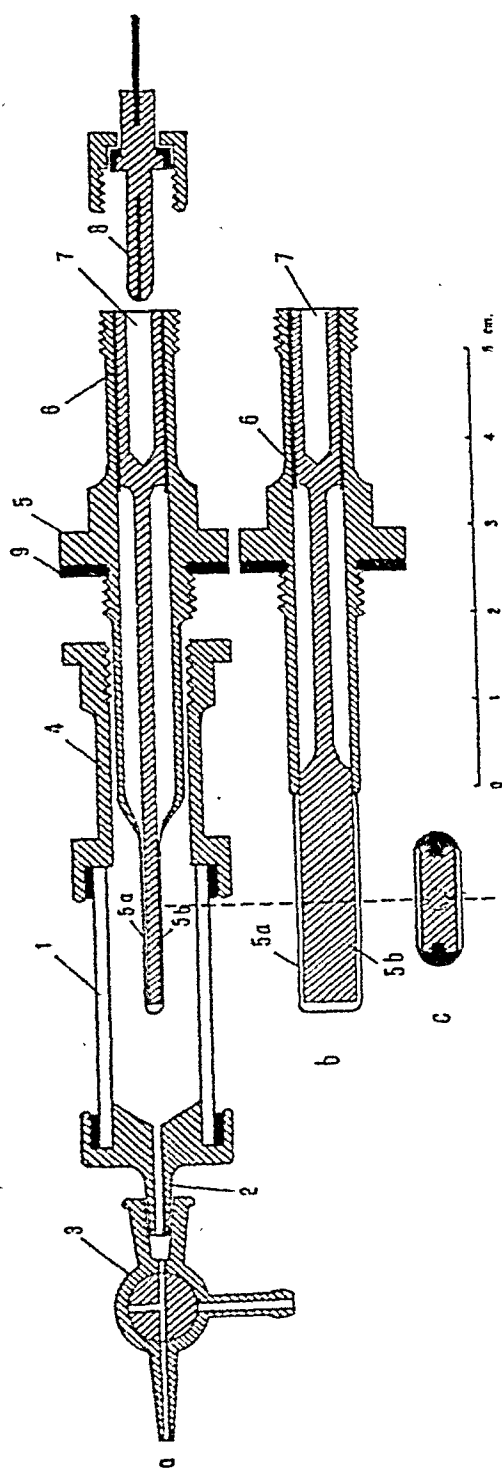


Fig. 1.

1. Cylindric glass tube (corresponding to injection syringe).
  2. Nozzle for the cannula.
  3. Three-way tap.
  4. Metal barrel of the syringe, into which the condenser chamber is screwed down.
  5. Condenser chamber (see 1 b).
  - 5 a. Longitudinal section.
  - 5 b. View of condenser chamber turned 90° about its longitudinal axis.
  6. Insulation.
  7. Socket for plug.
  8. Plug, with revolving nut.
  9. Rubber washer.
- c. Section through condenser chamber, showing the central condenser plate (5 b) surrounded by mica insulation.



An apparatus both physiologically and clinically serviceable for measuring the intra-arterial pressure in man must satisfy the following requirements:

1) It must be sufficiently sensitive and have an adjusting period sufficiently short to give a constant sensitivity up to 80—100 oscillations per second. The damping of the manometer must correspond to an approximately aperiodic adjustment, so that there will be no need for the introduction of any kind of additional damping into the system, and it must not show mechanical hysteresis or elastic after-effect; that is, it must possess constant sensitivity both statically and dynamically.

2) The transmission by which the pressure variations are registered must be such that the device registering the pressure is small and light and *can be easily applied independently of the recording part of the apparatus.*

In the system used by us, the variations in pressure cause a deformation and thereby a change in capacity of a "condenser chamber" consisting of a rectangular condenser plate  $1 \times 4 \times 20$  mm in size, placed isolated, in a closed box of phosphor bronze (the other plate of the condenser; see fig. 1.) The box fits closely around the plate, but without touching it, the distance between the two being about 10 to 20  $\mu$ . A variation in the pressure on the walls of the box, which are 0.2 mm thick, will cause a change in the distance between the latter and the plate, resulting in a rather considerable alteration of its capacity, even with very slight movements.

The condenser chamber is placed in a glass cylinder of the form of an ordinary 1 cm<sup>3</sup> injection syringe, and is fastened to a bottom piece which takes the place of the piston. The syringe is filled with sodium citrate to prevent coagulation, care being taken to avoid air bubbles



Fig. 2.

Cable. 1. Conductor. 2. Paper insulation. 3. Rubber tubes. 4. Electrostatic metal screen. 5. Protecting rubber tube.

The chamber and the syringe, being respectively of metal and glass, can be easily cleaned and sterilised. The condenser chamber is connected with a screened cable, the core of which connects with the central condenser plate; while the screen is in contact with the box. The connection is made by means of a plug with a revolving nut, and is thus easily made or interrupted as required.

The greatest difficulty has been to make this cable sufficiently light and flexible, but at the same time of low and constant capacity. A cross-section of it is shown in fig. 2. The conductor in the centre of the



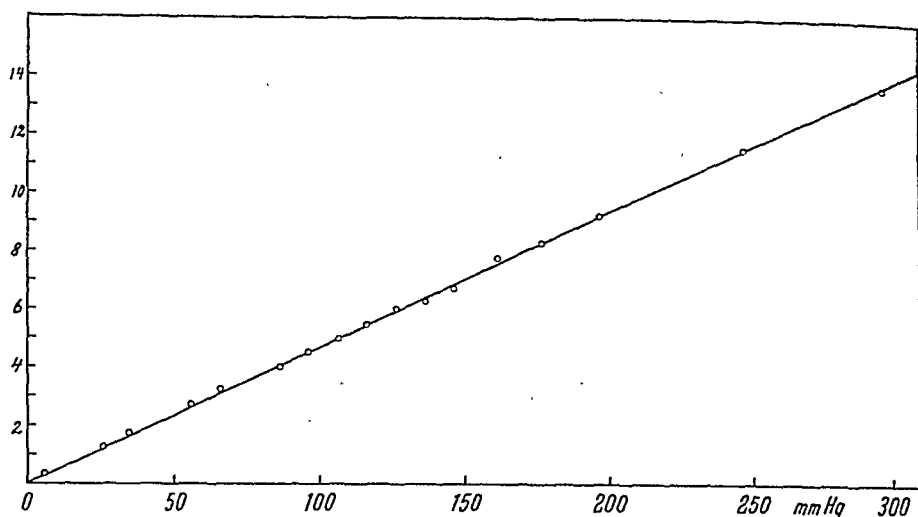


Fig. 4.

Capacity variation, in relative units, as function of the static pressure. — Ordinate: Excursion of the manometer (in relative units). — Abscissa: Pressure (in mm Hg.).

the sharpest bends of the cable cause an error which may amount to as much as 10 per cent of the mean pressure, corresponding to 10 mm Hg. Generally, the error is from one-fifth to one-fourth of the maximum value.

Between the injection cannula and the syringe there is inserted a small three-way tap (fig. 1), by means of which the condenser chamber can be connected to the atmosphere and thus register zero pressure. This is done several times in the course of each experiment. Variations in the capacity of the cable and possible drift in the amplifier will therefore be of no significance.

The sensitivity of the high frequency arrangement can be varied at will. A switch is built in, by means of which different small capacities corresponding to suitable pressure variations may be coupled in as control for the sensitivity.

The tension from the high frequency arrangement is directly connected to an a. c. amplifier with suitable time constant, e. g. as used in amplifier-electrocardiographs. Normally, only the pressure *variations* are registered in this way; but as an instantaneous fall of pressure to zero, which is registered, can be obtained by opening the three-way tap just mentioned, the height of the *actual* pressure can be measured, the registered fall representing the difference between the (intra-arterial) pressure *before* and the (zero) pressure *after* the tap has been opened.

For simultaneous, direct measurement of the pressure variations and the height of the resulting pressure a d.c. amplifier is used (BUCHTHAL and NIELSEN, 1936). With an electrostatic oscillograph as re-

gistering instrument, an amplification of 50 times (a single stage) is sufficient.

In a series of experiments not only the pressure variations, but also the mean pressure was measured. This was done with the aid of a low-pass filter in the d. c. amplifier, whereby an electrical integration of the pressure curve is obtained.

*The physical properties of the manometer.* — The static, capacitive sensitivity of the manometer is 0.01 cm per 100 mm Hg. As it will be seen from the curve in fig. 4, the measuring system is linear within a range from 0 to 300 mm Hg. The curve in Fig. 5 shows the sensitivity for low positive and negative pressures. The interdependence of pressure and capacity is reversible within the limits of accuracy set by the electrical measuring arrangement. The sensitivity of the

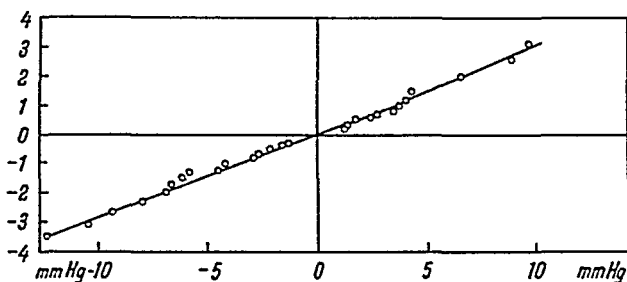


Fig. 5.

Static calibration for small negative and positive pressures. — Ordinate: Excursion of the manometer (in relative units). — Abscissa: Pressure (in mm Hg.)

pressure-registering device does not change with time, and variations in temperature are practically without significance.

The dynamic sensitivity of the pressure-registering device is equal to the static up to several thousand oscillations per second. When the condenser chamber is placed in fluid, in connection with a long, thin cannula, its dynamic sensitivity of course diminishes, because the fluid must move rapidly back and forth in the tube in order to deform the walls of the pressure chamber. With a given registering device possessing the above mentioned properties, the range of frequency within which true pressure values are registered is thus determined exclusively by the length of the cannula and the size of its lumen. The cannula must therefore be as short and have as wide a lumen as practically possible. In the experiments here described we generally used a cannula 20–25 mm long, with a bore of 1 mm.

The dynamic calibration was done by means of an air pump producing up to 150 strokes per second. The pressure-registering system — i. e. the syringe with condenser chamber, the tap and the cannula — are filled with fluid and placed in communication with the cylinder of the pump via a small air chamber. To maintain a constant mean pressure and constant variation of the pressure, the valves of the pump were closed, whereupon the pump in a few seconds was brought up

to a maximum of revolutions and at the same time the pressure variations registered. In this manner the dynamic sensitivity as a function of the frequency was ascertained for frequencies from about 5 to 210 vibrations per second (figs. 6 and 7). Without cannula the resonance frequency is about 120 vib./sec., (limited by the nozzle of the syringe),

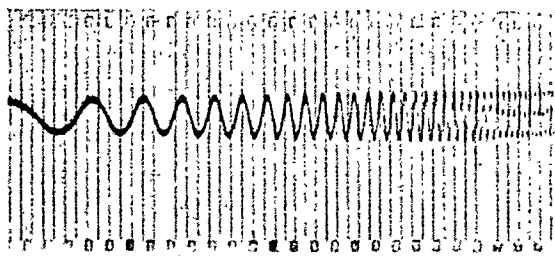


Fig. 6.

Dynamic calibration of the fluid-filled manometer with cannula. (Bore of cannula, 1 mm.; length 25 mm.). Periodically varying pressure of varying frequency. — Time marks, 20 ms.

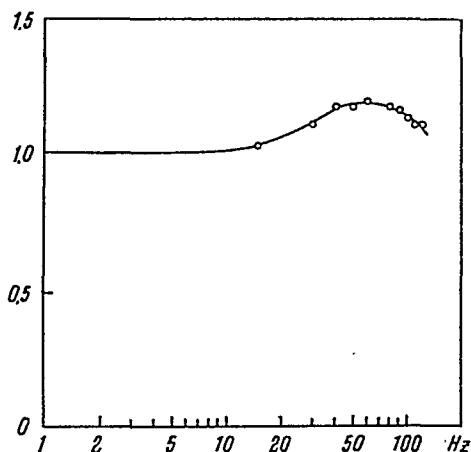


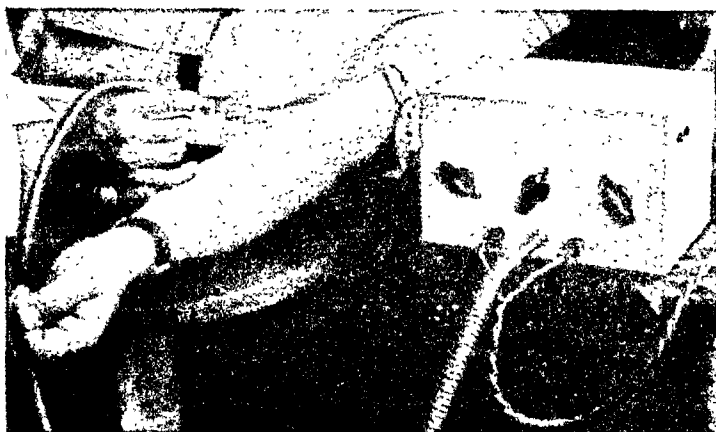
Fig. 7.

Dynamic calibration. Pressure excursions, in relative units, as function of the frequency (cf. fig. 6). — Ordinate: static sensitivity. — Abscissa: frequency (log. scale).

with a cannula 25 mm long and 1 mm in diameter about 90 vib./sec. Higher frequencies are registered with diminished sensitivity. The damping of the system is due chiefly to the fluid resistance in the cannula and is approximately aperiodic. When measuring arterial pressure it is therefore not necessary to introduce an air chamber into the system, which would affect the dynamic sensitivity.

In the following we shall relate experiments, some made with one and others with two of the manometers described, on the

brachial or femoral arteries of normal individuals, and some orientating experiments on patients with cardiac affections (35 persons in all). Comparison with the pressures found by the auscultatory method showed that there were more or less considerable differences between these and the values recorded by the manometers; the systolic pressure being in most instances 10 to 30 mm higher by indirect measurement. This accords with the experience of VON BONSDORFF (1932). The directly measured values for the diastolic pressure had no constant relation to those



*Fig. 8.*

The manometer connected with the high frequency arrangement for measuring the pressure in the brachial artery.

found by indirect mensuration, but with the latter the amplitude of the blood pressure variation is nearly-always measured too low. The experiments were made with the subject in recumbent position and the manometer placed about at the height of the heart. The pressures in the brachial artery were taken 1—2 cm above the elbow joint, at the medial margin of the biceps muscle (fig. 8); those in the femoral artery in the inguinal fossa, below the inguinal ligament. In most of the experiments the cannula was inserted into the artery at right angles to the latter, so that the measurement was truly a record of the lateral pressure. Control experiments with corresponding current velocities, both on man and on models, showed however that the error resulting from longitudinal insertion of the cannula into the vessel does not exceed 5 mm Hg. After the termination of the experiment the artery is compressed for about twenty minutes.

In fig. 9 a and b are shown the simultaneous registrations of an electrocardiogram and the pressure curves from the brachial and femoral arteries.<sup>1</sup> The time interval between the peak of the R wave and the point where the pressure begins to rise is for measurements in the brachial artery about 130 milliseconds

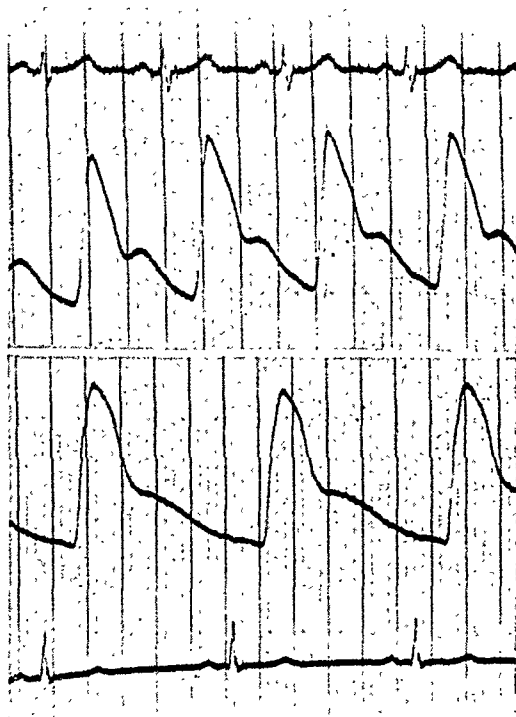


Fig. 9.

- a) Record of pressure in the brachial artery, together with electrocardiogram.  
 b) » » » » » femoral » » » » »

(ms.), for measurements in the femoral artery about 160 ms. When the pressure is taken simultaneously in the brachial and the femoral arteries (fig. 10), there will usually be a corresponding interval between the starting points of the curves, because the rise in pressure in the brachial artery begins 20–30 ms. before the rise in the femoral artery. The pressure also reaches its maximum later in the femoral artery, owing to the longer

When registering electrocardiogram and blood pressure changes simultaneously with an ordinary amplifier-electrocardiograph it is necessary to insulate the high-frequency arrangement from the patient in order to prevent escape of the electrocardiogram in the blood pressure curve. This is easily done by insulating the condenser chamber in the syringe.

distance over which it has to be propagated and the resulting smoothening out of sharp pressure impulses. The rising time is about 20 ms. longer in the femoral than in the brachial artery.

Besides the primary change in pressure there is, as we know, in the brachial artery first a slight, then a strong secondary wave; whereas in the femoral artery there is only one, strong secondary wave. The mechanism of the origin of these secondary waves is still debated and may be briefly mentioned here in con-

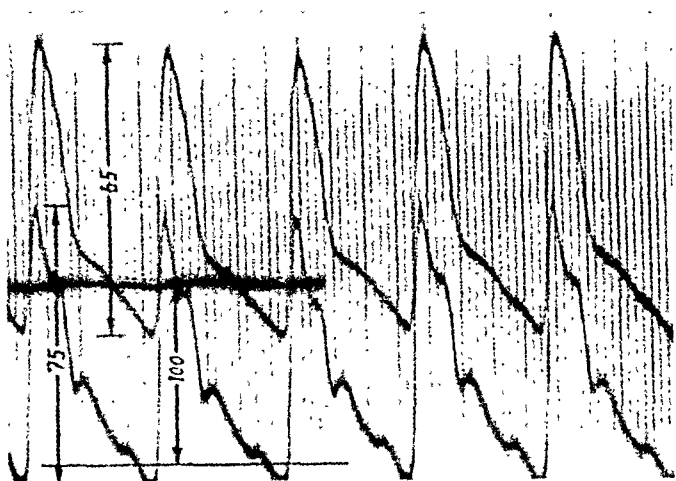


Fig. 10.

Simultaneous registration, with two manometers, of the pressures in the brachial (lower curve) and femoral (upper curve) arteries. The figures on the curves indicate the values of the mean pressure and the pressure variations in mm Hg. Time marks, 50 ms.

nection with the measuring of intra-arterial pressures during local occlusion immediately below the point where the pressure is taken. The artery is compressed by application of a strong pressure against the humerus, and can be closed completely in persons where there is not too much adipose tissue. When the occlusion is complete, the amplitude increases 15 per cent, and at the same time the mean pressure rises 10—15 per cent. The latter determination is less exact, however, owing to the delay when the mean pressure is registered. Both with and without occlusion the pulse curve from the brachial artery shows, after its maximum, besides the primary change in pressure, the two extra waves mentioned. The theories regarding the origin of these vibrations are discussed at length by VON RECKLINGHAUSEN



(1940). The first secondary wave occurs 150 ms. after the curve has reached its maximum, the second 250—300 ms. after the peak. Without occlusion, the amplitude of the first vibration is slight, but during occlusion it becomes markedly higher, and the first vibration therefore just as strong as the following (fig. 11).

The amplitude of the second vibration is not altered by the occlusion, but the curve gets a different form. During occlusion this vibration begins more steeply and sometimes has a double peak. The distance between these two highest points is less

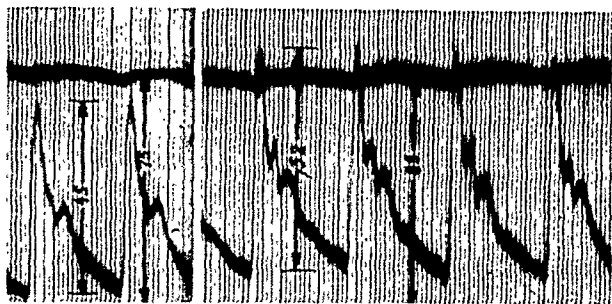


Fig. 11.

Alternating and mean pressures in the brachial artery under local compression distally to the place where the pressure is taken; a) without compression; b) local compression. The figures on the curves indicate the values of the mean pressure and the pressure variations in mm Hg. Time marks 50 ms.

than 50 ms. The increase in the amplitude of the first secondary wave during occlusion occurs without any simultaneous change of its location in relation to the peak of the curve, and must be due to the altered conditions of reflection and absorption at the point where the artery is compressed.

The pulse wave probably becomes reflected at the point of compression, and passing back to where the brachial artery joins the larger vessels is reflected there as a negative pressure wave, which is registered 150 ms. after the curve has reached its highest elevation. That the wave occurs even when the artery has not been compressed must be due to reflection at the point where the brachial artery divides, or at the branching-off of yet more distal ramifications. The latter is less likely, though; because the difference in time between the pressure maximum and after wave is not measurably changed by the occlusion. If the reflection occurred in the smaller branches of the artery this difference

would be increased, due to the propagation time over the increased distance.

The other possibility is that the wave really starts proximally to the point of compression but normally diminishes owing to the damping in the vessels distal to that point, because the entire elastic system must be considered as a unit. The absorption should then be hindered by the compression, and the vibration would occur in its actual size. The experiments described in the following, with measurement of the pressure during inhalation of amyl nitrite, indicate, however, that the wave is not initiated proximal to the point of compression; because it disappears under the influence of that drug, which should not be supposed essentially to affect the capacity for transmission and reflection of the valves or root or large branches of the aorta (though in this connection the velocity of the pulse wave plays no rôle).

The second after-wave — the much discussed dicrotic vibration — was originally thought to be determined by the closing of the aortic valves, an idea which corresponds also with the time of its occurrence. But as shown by VON RECKLINGHAUSEN, among others, the matter is considerably more complicated. The maximum pressure during diastole is due to the resulting reflection of the pressure wave through the whole vascular system. The second wave (the dicrotic vibration) is mainly this reflected wave ("Gewogewelle"), which becomes further deformed during the closing of the aortic valves, because the oscillating system has other elastic constants after closing than before.

A noticeable feature is the different course of the curve according to whether the measurement is taken from the brachial or the femoral artery (fig. 10). That the vibration is more pronounced in the former than in the latter is probably due to stronger reflection and better conditions for transmission in the brachial artery. A lesser reflection in the femoral artery is probable, because the vessels in that region are so large that the effect of an air chamber becomes more pronounced, which is also shown by the experiments under inhalation of amyl nitrite described below.

As we know, the mean arterial pressure falls during *inhalation of amyl nitrite*. At the same time the amplitude of the blood pressure diminishes, the frequency of the pulse increases and the form of the curve alters. With regard to the alteration of the curve the statements in the literature differ, probably because of the too great inertia of the measuring systems and owing to

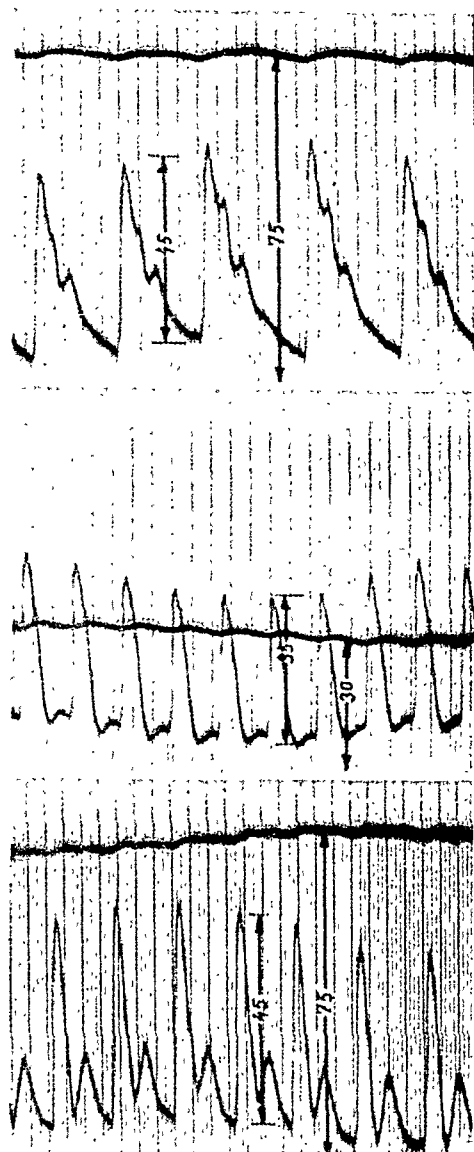


Fig. 12.

Pressure variations and mean pressure in the brachial artery during inhalation of amyl nitrite.

a) Before inhalation. b) 1.5 min. after the beginning of inhalation. Considerable decrease of mean and alternating pressure. c) 5 min. after cessation of inhalation. Mean pressure normal, but the curve of the alternating pressure still altered. The figures on the curves indicate the values of the mean pressure and the pressure variations in mm Hg. Time marks 50 ms.

the different moments during the action of the amyl nitrite at which the comparisons have been made. VON KRIES (1892) finds that the dicrotic vibration disappears and, in contrast to HOORWEG (1890) and TIGERSTEDT (1922), takes this as proof of its being set up peripherally; while other investigators (CUSHNY, 1925) find that the second after-vibration becomes more pronounced under the effect of amyl nitrite.

In our experiments with inhalation of the drug, the first of the secondary waves in the curve for the brachial artery disappears entirely, whereas the second remains, with the same distance in time from the beginning of the curve, *but apparently with considerably greater amplitude* (fig. 12). The first, sharp secondary vibration disappears owing to the changed conditions of reflection in the periphery; the highly dilated vessels giving only slight reflection of sharp impulses, because the air chamber effect is increased (absorption). The secondary vibration, which is mainly due to the summated reflection in the arterial system, is

not noticeably altered; but the systolic pressure falls more rapidly, owing to the lower peripheral resistance, and is lower at the moment when the reflected wave sets in; wherefore *it appears higher* than before the application of amyl nitrite. At the end of the experiment, when some of that effect is still present in the form of increased pulse rate, while the blood pressure has again reached its normal value, the form of the curve is still altered. The vessels still offer less resistance to the flow than normally; but the heart rate and the minute volume are larger than before the action of the amyl nitrite. LINDHARD (1915) and NEUKIRCH (1938) found that the minute volume was increased during the action of the drug, when the blood pressure was normal. The systolic pressure thus falls more rapidly than normally, and the reflected wave therefore becomes steadily more apparent.

The pressure variations in the femoral artery during the action of amyl nitrite are similar to those observed in the brachial artery; the fall in pressure becoming more rapid, but sometimes not rapid enough to further accentuate the reflected wave, which is already less pronounced here.

Simultaneous registrations of the electrocardiogram and the arterial pressure in the brachial artery during the action of amyl nitrite show a variation of the distance in time between the peak of the R wave and the start of the pressure rise. An increase of this distance during inhalation of the drug amounting to as much as 40 ms. has been ascertained. It may be due either to a change in the mechanical latency of the heart or, more probably, to a change in the rate of the propagation of the pulse wave.

### Summary.

A new manometer is described with electric transmission for direct mensuration of the intra-arterial pressure in man. Its static and dynamic sensitivity is such that all the details of the course of the curve are reproduced without distortion, both as regards time and amplitude. The pressure variations act on a condenser chamber and are registered as changes of capacity.

As examples of its use experiments are described with simultaneous registrations of the intra-arterial pressure and the electrocardiogram, and with simultaneous registrations of the pressures in the brachial and femoral arteries. Besides, the arterial pressure

was measured under the effect of amyl nitrite and local occlusion distal from the point where the pressure was taken. The various components of the pressure curve, and their variations under these conditions, are discussed.

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## On the Enzymic Hydrolysis of Some Lactones.

By

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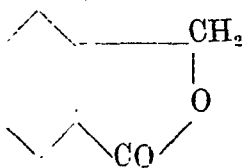
Received 1 October 1942.

The enzymic hydrolysis of ordinary esters has for many years been investigated with great thoroughness, and to a certain degree of completeness in some directions. As a consequence of this a very large body of experimental material has been accumulated. Now the question rather naturally presents itself, how the *inner* esters, the lactones, behave under enzymic hydrolysis. However, this problem has not received much attention in the literature. There is a paper by BAMANN and SCHMELLER (1931) on the subject, in which  $\gamma$ -valero lactone,  $\gamma$ -butyro lactone, cumarine and santonine are investigated. Their method was essentially the titrimetrical method of KNAFFL-LENZ (1923), with the precautions as regards indicators which were necessitated by the investigations by the same authors on the poisoning action of indicator dyes on esterases (1931). The result was that the enzymic hydrolysis was found to be vanishingly small for all the lactones mentioned above. Furthermore, if one of those lactones was added to a reaction mixture containing methyl butyrate, the enzymic hydrolysis of the latter ester was very considerably decreased. This retardation may, according to BAMANN and his collaborators, be ascribed to the formation of a rather firmly bound complex between esterase and lactone. The results are obviously of great interest, since, if they can be substantiated and generalized by further work, they would seem to express a general chemical property of the esterase molecule, which may perhaps some day contribute essentially towards a deeper understanding of esterase activity.

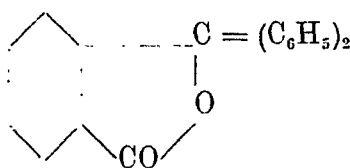
The experimental material so far available on the hydrolysability of lactones by esterases is rather meagre, being restricted to four cases only, and does not allow of convincing generalizations. It was therefore decided to investigate some more lactones, with a view to proving that the effects described above are really expressing characteristic properties of lactone rings and esterases, and are qualitatively independent of side conditions, as, e. g., the presence of other functional groups in the lactone molecule, and the hydrogen ion concentration in the reaction mixture (at least in the alkaline range). It should also be shown experimentally that the results are independent of the particular ester used in the retardation experiments.

We have selected for investigation the following lactones (or compounds containing a lactone ring):

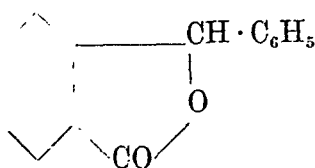
*Phtalid* (the lactone of o-oxy-methyl-benzoic acid)



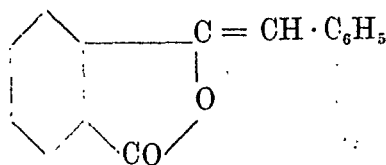
*Diphenyl phtalid*



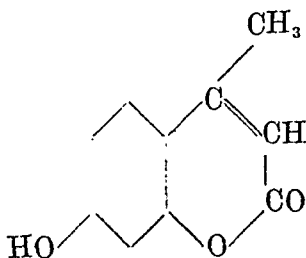
*Lactone of o-benzhydryl benzoic acid*



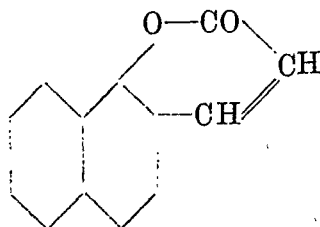
*Benzal phtalid*



*4-methyl umbelliferon*



*Cumarin of  $\alpha$ -naphthol*

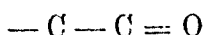


The method applied was, on account of its simplicity and rapidity, the titrimetrical one of KNAFFL-LENZ (l. c.). As indicators

we have used brom cresol purple, neutral red and phenol phtalein. The first two are recommended by BAMANN and SCHMELLER (l. c.) in their investigation on the applicability of the KNAFFL-LENZ method. Phenol phtalein was used in order to show that the effects observed do not depend qualitatively on the hydrogen ion concentration of the reaction mixture (at least in the alkaline range), or on the nature of the particular dyestuff used as indicator. For the same purpose a few experiments were also carried out with brom thymol blue.

The results may be briefly summarized as follows: All the lactones investigated were found to be almost non-hydrolysable by the esterases of the liver of the pig. Furthermore, when the lactones were added to an emulgaion of tributyrin, the hydrolysis of this latter compound was very considerably retarded. The same effects were obtained in a few experiments with the diethyl esters of fumaric and maleic acids. It would seem justified to conclude that both the effects observed, the non-hydrolysability and the retardation, are rather general properties of the liver esterases.

A theoretical explanation and interpretation of these findings are at present hardly possible, in view of our ignorance about the chemical constitution of the esterase molecule. It is tempting, however, in accordance with BAMANN and SCHMELLER (l. c.) to ascribe the effects to the property of the group



of the lactone ring to form particularly firmly bound complexes with the esterase molecules.

### Experimental part.

*Substrates.* The preparation of the various substrates already mentioned above was carried out as follows:

Phtalid was prepared according to REISSERT (1913).

Diphenyl phtalid was obtained by a Friedel-Crafts reaction according to BAEYER (1880).

o-benzoyl benzoic acid was prepared according to HELLER and SCHÜLKE (1908), and from this the lactone of benzhydryl benzoic acid was obtained following a method of ULLMANN.

Benzal phtalid was prepared by the procedure described in Organic Synthesis 13.10, which is essentially identical with the method of GABRIEL (1885).



4-methyl umbelliferon and the cumarin of  $\alpha$  = naphthol were both prepared by the methods described for instance by SIMONIS (1916); they are essentially due to VON PECHMANN and to BARTSCH.

The tributyrin used was a commercial product, purified by redistillation.

*Enzyme solution.* The experiments were carried out with a glycerol extract from pigs liver. The organ, from a newly killed animal, was cut into slices, and forced twice through a meat mill, after which it was carefully ground in a mortar with sea sand. It was then extracted with glycerol for several hours, and carefully filtered. The extract, when stored in the ice box, retained its activity for a fairly long period of time. As is well known, these simple solutions have the advantage of a low protein content.

The KNAFFL-LENZ method is so well known that we shall not describe its details here, except to note that care was taken to utilize the results of BAMANN and SCHMELLER referred to above. According to these authors brom cresol purple and neutral red can be used without in any way endangering the applicability of the method. In some of our experiments phenol phtalein was also used.

All measurements were carried out in the temperature interval  $20^{\circ}$  &  $0.1^{\circ}$  C.

*Enzymic hydrolysis of lactones.* The experiments were carried out by suspending 0.2 g of the lactone in 100 cm of water, and adding the enzyme solution and the indicator. For the latter both brom cresol purple, phenol phtalein and neutral red were used. The duration of the experiments was  $1\frac{1}{2}$  hours. In no case could any enzymic hydrolysis be detected.

*Retardation experiments.* These experiments were performed by suspending or emulgating 0.2 g tributyrin in 100 cm<sup>3</sup> of water, and then adding the enzyme solution, the indicator and 0.15 g of the lactone under examination. Comparison experiments without lactone were carried out to determine the rate of hydrolysis of tributyrin itself. The self-hydrolysis was also determined and taken into account. All titrations were carried out with  $n/10$  NaOH, and the duration of the experiments was  $1\frac{1}{2}$  hours. The results of typical experiments may be summarized in the following table.

$\text{Cm}^3 \frac{n}{10} \text{ NaOH added after } 1\frac{1}{2} \text{ hours.}$

Substrate \ Indicator	Brom caesol purple	Phenyl- phtalin	Neutral red
Tributyrin . . . . .	7.5	14.7	7.5
Tributyrin + phtalid . . . . .	6.6	9.7	5.9
" + diphenylphtalid . . . . .	6.2	11.4	5.7
" + benzalphtalid . . . . .	5.9	7.4	5.2
" + lactone of o - benz- hydryl benzoic acid . . . . .	4.8	11.7	5.0
" + 4 - methyl - umbelli- feron . . . . .	5.2	7.8	5.1
" + cumarin of $\alpha$ - naphthol	5.7	12.0	6.5

It should be clear from this table that qualitatively there can be no doubt about the results.

Some experiments were also carried out

a) with both shorter and longer duration of the experiments,

b) with varying relative amounts of enzyme solution, lactone and substrate,

c) with brom thymol blue as indicator,  
and finally also

d) with the diethyl esters of fumaric and maleic acids as substrates instead of tributyrin.

The results were qualitatively the same in all cases, and we therefore feel justified in refraining from the reproduction of further numerical data. As regards b) our results were closely similar to those found by BAMANN and SCHMELLER (l. c.); by decreasing concentrations of the ester, one and the same amount of lactone caused increasing retardations of the hydrolysis.

Since the lactones used are only slightly soluble in water, it might be thought that the phenomena observed were at least partly due to some adsorption mechanism. To test this point we carried out some experiments using for instance anthraquinone instead of the lactone. No significant differences in the velocity of hydrolysis were observed in this case, and the conclusion is therefore drawn that we are in all probability dealing with a true chemical phenomenon.

The author wishes to express his thanks to Professor E. BILMANN and Prof. R. EGE for their hospitality. It is also a pleasure to thank Lektor ST. VEIBEL for his interest in this work and for several discussions.

### Summary.

An investigation is made of the hydrolysis by liver esterase of some lactones, which are found to be practically non-hydrolyzable. It is further found that these lactones retard the hydrolysis of esters. The interpretation of these findings is briefly discussed.

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## **A Method for Detection and Determination of Carbon Monoxide in Coagulated Blood.**

By

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In a previous paper (WENNESLAND 1940) I have described a new method for the detection and determination of carbon monoxide in blood. Since the publication of the paper I often receive samples of blood for analysis. It happens that the blood has coagulated, it may be that he who has taken the sample, is not always aware that for analysis of carbon monoxide the blood ought to be unclotted, or the sample has been taken from a dead body. I thought it to be of practical interest if my method could be modified for analysis of such coagulated blood. Supported by the fact that the colloid contents of plasma did not seem to increase the diffusion time of carbon dioxide from plasma to an absorbing fluid, as analyses of plasma carbon dioxide showed an identical curve for reaction time compared to carbon dioxide analyses of a solution of sodium carbonate in water (WENNESLAND 1941), and by the observation that even when the colloids of the blood had been precipitated to form a gel, the diffusion of carbon monoxide was as rapid and complete as when no gel was formed (WENNESLAND 1940), I supposed that perhaps the carbon monoxide of coagulated blood could also be completely driven out by the addition of sulfuric acid, and determined by the principle previously described (1940).

The results of the experiments showed that this was possible. and with a slight modification of the method, I am able to tell within 5 to 30 minutes after I have received a sample of clotted

blood, whether it contains large or small quantities of carbon monoxide, and I can determine quantitatively the contents of the gas as rapidly and accurately as in unclotted blood.

### Principle.

The volume of the clotted blood sample is measured, the clot is crushed, and the whole sample is distributed in portions into Erlenmeyer flasks. The carbon monoxide is liberated by converting the hemoglobin into acid hematin. Each of the flasks is connected by means of an airtight rubber cuff with a corresponding one, which contains a known solution of palladium chloride. The carbon monoxide liberated, passes into the palladium chloride and reduces an equivalent quantity of the latter to metallic palladium, which appears as a dark brown metallic mirror on the surface. By observing the time which has passed from the addition of the acid until the metallic palladium appears, and by judging the solidity of the mirror formed, one can roughly estimate the quantities of carbon monoxide present.

When reduction has reached a maximum, excessive palladium chloride can be determined by a simple iodometric procedure described by the author (1940). Original palladium chloride is determined in a blank, and the content of carbon monoxide is calculated from the difference.

*Reagents* and equipment have been described previously (1940). To measure the sample and crush the clot one needs an accurately graduated syringe with close fitting piston.

### Procedure.

When great accuracy is wanted, the blood ought to be handled strictly anaerobically. It is poured into the syringe, the piston is set in place, and the volume is read on the gradations of the syringe, when all air has been expelled.

Then the blood is delivered by squeezing it through the tip of the syringe in portions of 1—2 cc. into an Erlenmeyer flask of 50 cc., which contains 2 cc. of distilled water. The clot is thereby crushed and finely distributed.

About 0.5 cc. of 10 per cent sulfuric acid is delivered from a burette into the blood — water mixture, and the flask is connected as quickly as possible with the other one, which contains 2 cc.

of a 1/100 N solution of palladium chloride. When the rubber cuff is moistened with a little water, it glides easily and smoothly over the neck of the flasks. The latter are kept in a horizontal position, rolled a little on the table to mix blood and acid, and left lying still, or placed in the rotator described by the author (1940).

### Detection of Carbon Monoxide.

When only detection of carbon monoxide is of interest, e. g. for diagnostic purpose, one observes the time which passes from the addition of sulfuric acid until a metallic mirror is seen on the surface. A few minutes later the mirror becomes solid, and with a little practice one can roughly estimate the contents of carbon monoxide by judging the solidity of the mirror formed.

For clinical use I have arranged the observations in Table I.

The data are identical whether clotted or unclotted blood is examined.

Table 1.

#### *Detection and Rough Estimation of Carbon Monoxide in Blood.*

About 1 cc. of blood in each set of flasks. All values are approximate, and are applicable whether clotted or unclotted blood is examined. Present method.

Contents of CO		The flasks were left lying still		The flasks were rotated	
Vol. per cent	Per cent of hgb. saturated	Pd. mirror was visible after:	Mirror was solid after:	Mirror visible after:	Mirror solid after:
		Minutes	Minutes	Minutes	Minutes
1	5	30	—	15—20	—
2	10	20	25	10—12	15—20
4	20	15	20	10	15
8	40	12	15	8	10
12	60	9	10	6	10

### Determination of Carbon Monoxide.

If one also is interested in accurate determination of carbon monoxide, the reaction must proceed until the reduction of palladium chloride has reached a maximum. When the flasks have been left lying still, 14—18 hours are needed; when rotated, maximum occurs after 3 hours. (Author 1940 and Fig. 1 of this paper.) The curve of Fig. 1 is seen to be identical with the curves of Fig. 3 of the paper of 1940.

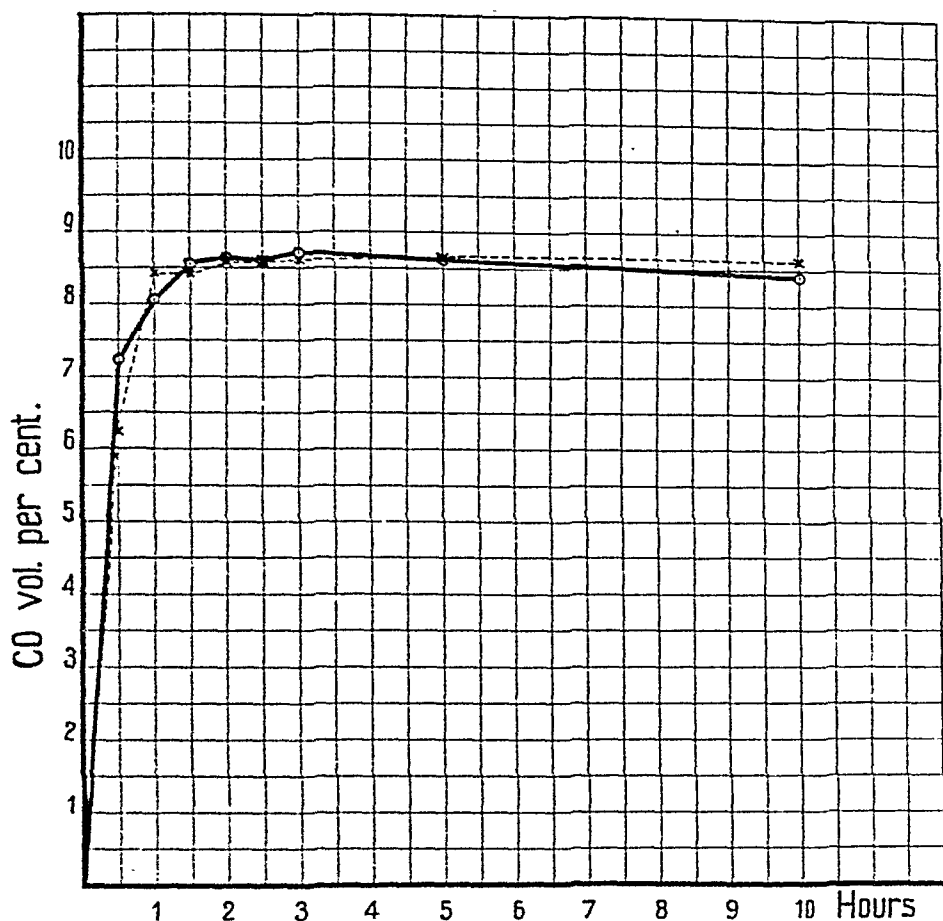


Fig. 1. Analyses of blood for carbon monoxide.

Empirical curves showing the correlation between analysis values and rotation time for clotted blood ———, and for unclotted samples of the same blood - - - - -.

When maximum reduction has taken place, the flasks are disconnected, and the further iodometric determination of non-reduced palladium chloride is performed as shown previously (1940) with a small modification: After addition of 10 cc. of 1/400 N potassium iodide into each of the palladium chloride solutions and vigorous shaking, the contents of the flasks are filtered through the same white ribbon filter paper into a common clean and dry 100 cc. Erlenmeyer flask. The common filtrate is shaken to be uniform, and gives a mean value of excessive palladium chloride after reduction by the carbon monoxide of the whole sample.

Aliquot portions of 5 cc. are taken and analysed in the ordinary way: Oxidation of KI to  $\text{KIO}_3$  by excess of bromine, which is

removed a minute or two later by 2 cc. of a mixture of 2.5 per cent phenol with 2.5 per cent sulfuric acid added from a burette. Iodine is liberated from the  $\text{KIO}_3$  by addition of KI in excess, and is ultimately titrated against 1/200 N solution of sodium thiosulfate. The number of cc. of thiosulfate added, gives us the  $b_2$  of the formula for calculation.  $b_1$  is found by titration of the original 2 cc. of palladium chloride solution in the same way, or better by titration of a blank, which gives correction for carbon monoxide contingently present in the laboratory air enclosed in the flasks.

The content of carbon monoxide in volume per cent of blood is calculated by the formula:

$$\text{CO vol. per cent} = (b_2 - b_1) \frac{\overline{F}}{\overline{n}} = (b_2 - b_1) F \frac{n}{\overline{V}}$$

F is found by Table V of the paper of 1940, when the concentration of the thiosulfate has been adjusted against 5 cc. of 1/200 N standard solution of potassium iodate, or by the formula there, by which F of the table has been calculated, valid for blood samples of 1 cc. As the average size of the coagulated blood sample of

each set of flasks is  $\frac{V}{n}$  cc., where V is the total volume of the sample,

n the number of flasks in which the sample has been distributed,

F must be divided by  $\frac{V}{n}$ , and the formula is modified as above.

Example: By titration of 2 cc. of the palladium chloride solution (or by a blank),  $b_1$  was found to correspond to 2.85 cc. of thiosulfate solution. The clotted blood had a total volume of 3.6 cc. and was distributed in two sets of flasks. By the final titration of aliquot portions of 5 cc. of the common filtrate,  $b_2$  was found to be 9.45 cc. of thiosulfate. For titration of 5 cc. of 1/200 N standard solution of potassium iodate 5.02 cc. of thiosulfate were required ( $y = 5.02$ ), and F was found in Table V (1940) to be 2.32. The content of carbon monoxide present would be:

$$\text{CO} = (9.45 - 2.85) \times 2.32 \times \frac{2}{3.6} = 8.51 \text{ vol. per cent.}$$

If I thought there might have been any possibility that the sample had half of its hemoglobin or more saturated by carbon monoxide, I would not have dared to divide 3.6 cc. in two portions only, as I would have run the risk of having too little palladium

chloride for oxidation of all the gas. 2 cc. of 1/100 N palladium chloride solution are capable of oxidizing 0.224 cc. of carbon monoxide (0°C 760 mm Hg.).

### Details.

The sample of blood may be examined in one portion, by using larger flasks of 100 cc. and adding 1—2 cc. of 10 per cent sulfuric acid. The second flask must contain  $2 \times 2$  cc. of  $\text{PdCl}_2$ , or  $2 \times 3$ ,  $2 \times 4$ , etc. depending upon the size of the sample and its possible content of carbon monoxide. Corresponding multiples of 10 cc. of potassium iodide solution must be added, and  $n$  of the formula above corresponds to the multiple applied.

One might expect a longer rotation time when the blood was not divided in small portions, but experience showed, that even a 5 cc. sample which contained about 7.7 vol. per cent of carbon monoxide had reached a maximum after 3 hours (Table II). As a routine procedure I prefer to divide the sample in proper portions and distribute it in more flasks. The setting of the flasks is simple and quickly done, and my rotator is made for 50 cc. flasks.

Table 2.

#### *Analyses of Clotted Blood for Carbon Monoxide.*

5 cc. clotted samples of the same blood were analysed undivided in one set of flasks. Rotation time varied from 3 to 6 hours. A 1 cc. sample of the same blood unclotted was taken for control. The method of the author in all analyses.

Rotation time	Contents of CO of the clotted blood	Contents of CO of control sample of unclotted blood
Hours	Vol. per cent	Vol. per cent
3	7.71	7.61
4	7.68	
5	7.78	
6	7.80	

The rotator may be driven by an improvised water wheel (AUTHOR 1941) and may be simplified a little by making the nicks so deep and narrow, that they keep the flasks in place without any rubber ribbon.

As to the final titration, I have observed that one some times runs the risk of adding too small excess of bromine. It may occur when the bromine water is freshly prepared and not completely



saturated, that the 3—4 drops which I generally use, may be insufficient. On the other hand it is generally warned against adding so much bromine that the excess will form a cloudy precipitate with phenol (e. g. CLOSS 1931).

I have added 15—20 drops of bromine water without observing any disturbing effect of the precipitate. Only the colour change of the starch was indistinct when the precipitate was too solid. It is advisable to add rather too much than too little bromine. With 6—7 drops I have never failed. With practice one can judge by the brilliant lemon yellow colour whether an adequate excess of bromine is present.

### Results.

I prepared large samples of blood by mixing at random a portion of carbon monoxide saturated blood with unsaturated. The sample was handled and stored anaerobically in a 50 cc. glass syringe, whose tip was provided with a small rubber cap made from a short rubber tube, which was closed at the other end by a rod (BARCROFT and HALDANE 1902, SCHOLANDER 1938).

Coagulation was prevented by 0.15 per cent of potassium oxalate. 5 cc. portions were pipetted into small volumetric cylinders under a layer of paraffine oil, and to each portion were added equivalent quantities of  $\text{CaCl}_2$ . The cylinders were stoppered so that no air could enter, and shaken vigorously. Coagulation occurred after a few minutes. I prepared 4—7 clots of each sample, and control analyses of 1 cc. of unclotted blood were taken from the syringe before, between and after the preparing of the clots.

The latter were distributed in 3—4 flasks and analysed as described. Out of the common filtrate of each clot were taken 5 aliquot portions for titration. Each titration was regarded as one observation for calculation of the standard deviation. The latter was transformed into coefficient of variation (the standard deviation as per cent of the mean observation).

The results are seen in Table III.

The fact that the diffusion of carbon monoxide seems to go as rapidly from clotted blood, and can be determined as accurately as in unclotted blood, indicates that the present method may be applicable for determination of the carbon monoxide contents or capacity of tissues. For devices to measure and crush the tissues I refer to SCHOLANDER (1940 and 1942).

Table 8.

*Analyses of Clotted Blood for Carbon Monoxide.*

5 cc. samples of clotted blood were examined and compared to control analyses of 1 cc. of the same blood unclotted. The method of the author applied in all analyses. The clotted samples were distributed in 3—4 sets of flasks.

	C l o t t e d   b l o o d				Unclotted blood
	Number of clots	Number of titrations	CO contents in vol. per cent	Coefficient of variation	CO contents in vol. per cent
Sample 1	5	25	7.70	0.9	7.72
"  2	6	30	7.66	0.8	7.67
"  3	4	20	7.54	1.0	7.59
"  4	7	35	5.46	1.0	5.44
"  5	7	35	7.18	0.9	7.12
"  6	7	35	6.48	0.6	6.46
"  7	7	35	6.85	1.0	6.40
Mean of all samples	43	215	6.91	0.9	6.91

**Summary.**

The method of the author for detection and determination of carbon monoxide in blood is modified for analysis of coagulated blood. The volume of the blood is measured in a graduated syringe, and the clot is finely crushed by squeezing the sample out through the syringe tip. It is delivered into Erlenmeyer flasks, and carbon monoxide is liberated by adding sulfuric acid. The flasks with blood acid mixture are connected by means of an airtight rubber cuff with other flasks, which contain a known solution of palladium chloride.

The carbon monoxide passes into the palladium chloride, and a fine dark brown mirror of metallic palladium is seen after 5 to 30 minutes. The reduction of palladium chloride reaches a maximum after 3 hours when the flasks are rotated, 14 to 18 hours when they are left lying still. Excess of unreduced palladium chloride can be determined iodometrically, and the quantity of carbon monoxide present is calculated from the difference between original and excessive palladium chloride.

The method can also be applied as an approximative one for detection and rough estimation of carbon monoxide, by observing the time elapsed from the addition of the sulfuric acid until the mirror of metallic palladium is visible, and by judging the solidity of the mirror.

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## Über die fiebererregende Wirkung des Adrenalins.

von

U. S. v. EULER, E. LINDER und S.-O. MYRIN.

Eingereicht am 10. Oktober 1942.

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PELLACANI beobachtete im Jahre 1879 dass Injektionen von Nebennierenextrakten in grösseren Mengen eine Temperatursenkung und in kleineren Mengen eine Temperatursteigerung an Versuchstieren herbeiführten. Diese Befunde sind nachher mit reinem Adrenalin mehrfach bestätigt worden. Bei direkter Applikation von kleinen Adrenalinmengen im Hirnventrikel fanden JACOB und RÖMER (1912) eine Temperatursenkung von  $0^{\circ}.5$  C., die einige Stunden anhielt. BARBOUR und WING (1913) machten ähnliche Befunde und nahmen eine direkte Wirkung am Nervengewebe an. CLOETTA und WASER (1916) erhielten eine lokale Temperatursteigerung von  $0^{\circ}.6$  im Frontalhirn nach intravenöser Injektion von 0.2 mg sowie auch nach Applikation von 1/15 mg Adrenalin in den einen Seitenventrikel. Nach einigen Minuten fing auch die Darmtemperatur an zu steigen. KONDO (1919) fand keine nennenswerte Temperaturwirkung von 0.05—1 mg pro kg Körpergewicht bei subkutaner, intravenöser oder intraperitonealer Injektion. Wenn dagegen 0.01—0.1 mg Adrenalin pro kg in einem Seitenventrikel injiziert wurde trat eine Temperatursteigerung ein die ihr Maximum nach 2—3 Stunden erreichte.

Um der Natur und den Bedingungen der Adrenalinwirkung auf die Körpertemperatur näher zu kommen, besonders mit Rücksicht auf die Bedeutung einer Hyperadrenalinämie bei infektiösem Fieber beim Menschen (EULER, 1927) haben wir Versuche angestellt, wobei Art der Zufuhr, Quantität und Einwirkungsdauer berücksichtigt wurden. Auch waren wir bestrebt, deutliche und konstante Effekte zu erzeugen, da frühere Untersucher meist bescheidene Wirkungen nachgewiesen haben.

## Methodisches.

Als Versuchstiere wurden Kaninchen von 2—2.5 kg benutzt. Das Adrenalinpräparat war die reine Base, die entweder als solche verabreicht wurde, oder mit der berechneten Menge Salzsäure zuerst in Lösung gebracht wurde. In anderen Fällen wurde eine käufliche Lösung von Adrenalinhydrochlorid benutzt (Exadrin, »Astra«), ohne dass ein Unterschied zwischen den letzten zu verzeichnen war.

Folgende Administrationsformen wurden benutzt: a) Subcutane Applikation einer »Tablette« die von Adrenalin bzw. Adrenalinhydrochlorid und Glukose bereitet wurde, b) subcutane Injection einer Suspension der Base in Olivenöl oder in 2.5 % Agar. c) Injektionen von Adrenalin als Base oder Hydrochlorid in Ringerlösung in den III. Hirnventrikel. Hierbei wurde in Lokalanästhesie oder kurzer Äthernarkose ein kleines Loch im Schädeldach gebohrt und ein mit Sperrvorrichtung versehene feine Injektionsnadel auf die vorher ausprobierte Höhe in der Mittellinie eingeführt. d) Injektionen in die Zisterna magna dicht unterhalb des Schädels. Als Kontrolle der richtigen Einführung der Nadel wurde das Austreten von blutfreier Zerebrospinalflüssigkeit benutzt.

Die Temperaturmessung geschah mit einem kontrollierten Thermometer der etwa 7 cm lang ins Rektum der Versuchstiere hineingeschoben wurde.

## Ergebnisse.

### Normaltemperaturen.

An drei normalen Kaninchen wurden die spontanen Variationen der Rektaltemperatur an 4 verschiedenen Tagen zu verschiedenen Zeitpunkten des Tages gemessen.

Tabelle I.

8 <sup>h</sup> —9 <sup>h</sup>	12 <sup>h</sup> —14 <sup>h</sup>	15 <sup>h</sup> —16 <sup>h</sup>	18 <sup>h</sup>
39.6	39.6	39.9	39.7
39.2	39.6	39.5	39.2
39.1	39.7	39.8	39.6
39.4	39.6	39.4	39.7
38.8	39.4	39.1	39.4
38.8	39.4	39.6	39.6
39.6	39.4	39.8	39.9
38.9	39.6	39.4	39.6
39.0	39.4	39.7	39.7
39.6	39.7	39.9	39.9
39.3	39.0	39.6	39.6
39.4	39.0	39.6	39.4
39.23 ± 0.09	39.48 ± 0.06	39.61 ± 0.07	39.61 ± 0.06

Die Werte schwankten somit zwischen 38°.8 und 39°.9. Temperaturen von 38°.7 und 40°.0 wurden jedoch gelegentlich beobachtet. Aus der Tabelle ist ersichtlich, dass die Morgentemperatur etwas niedriger liegt als die Nachmittagstemperatur.

### Subcutane Deposition von Adrenalin.

Die subcutane Einführung auch von grossen Dosen Adrenalinbase (als eine kleine »Tablette« mit Glukose) hatte keine sichere Einwirkung auf die Temperatur. So bewirkte 40 mg der Base eine Höchsttemperatur von 40°.1, die nach 6 Stunden gemessen wurde. An den folgenden 4 Tagen stieg die Temperatur nicht über 39°.7.

Wenn statt dessen Adrenalinhydrochlorid verwendet wurde, trat in einem Falle eine Temperatursenkung und nachfolgender Tod innerhalb 18 Stunden nach 15 mg ein, während in einem Fall keine sichere Wirkung auf die Temperatur zu verzeichnen war. Die maximale Temperatur betrug 40°.4. Da die preliminären Versuche somit keine oder nicht konstante Wirkungen aufwiesen, wurde zu anderen Administrationsformen übergegangen.

### Subcutane Injection von Adrenalin in Olivenöl oder Agar.

Bei dieser Zufuhrart waren die Wirkungen deutlicher. So bewirkte 9 mg Adrenalin als Base in 0.9 ml Olivenöl aufgeschwemmt zunächst eine kleine Temperaturerhöhung auf 40°.1 nach einer Viertelstunde. Innerhalb der nächstfolgenden 2 Stunden erfolgte der Tod des Kaninchens. Ein zweites Tier starb nach derselben Menge innerhalb 12 Stunden nach einer vorangehenden Temperatursenkung. Kontrolltiere, die nur 0.8 und 0.9 ml Olivenöl subcutan erhielten, zeigten keine sichere Temperaturvariationen, und ebensowenig hatten Kontrollinjektionen von 0.8 ml 2.5 % Agar, das in anderen Versuchen als Vehikel verwendet wurde eine Wirkung.

Tabelle II.

23.X.

24.X.

	7 <sup>30</sup>	11 <sup>15</sup>	14 <sup>30</sup>	16 <sup>30</sup>	17 <sup>00</sup>	17 <sup>15</sup>	17 <sup>30</sup>	19 <sup>20</sup>	20 <sup>45</sup>	7 <sup>30</sup>	14 <sup>45</sup>	19 <sup>00</sup>
I	39.4	39.4	39.6		39.1	9 mg Adr. in 0,9 ml Olivenöl subcutan	40.1	+				
II	39.1	39.5		39.5			40.0	38.5	37.9	+		
III	39.1	39.5		39.8		0,9 ml Olivenöl subkutan	40.0	39.5	39.5	39.7	39.9	39.9

Tabelle III.

	26.X				27.X				28.X		30.X
	7 <sup>30</sup>	13 <sup>00</sup>	14 <sup>30</sup>	15 <sup>30</sup> —16 <sup>00</sup>	20 <sup>30</sup>	7 <sup>30</sup>	12 <sup>00</sup>	14 <sup>00</sup>	19 <sup>00</sup>	9 <sup>30</sup>	11 <sup>15</sup>
I	39.3	40.0	39.9	4 mg Adr. in 0.8 ml Olivenöl subkutan	37.4	37.2	37.6	37.5	37.2	37.6	38.8
II	39.5	39.5	39.6	0.8 ml Olivenöl	39.1	38.8	39.0	38.9	39.2	38.8	38.9
III	39.4	39.5	39.2	4 mg Adr. in 0.8 ml 2.5 % Agar subkutan	36.6	38.7	38.9	39.4	38.5	38.2	38.9
IV	39.7	40.0	39.8	0.8 ml 2.5 % Agar subkutan	39.6	39.2	39.5	39.4	39.6	39.3	39.0

Mit Dosen von 4—5 mg Adrenalinbase in 0.8—2 ml Olivenöl starb ein Tier am selben Tage und ein Tier am zweiten Tage nach vorangehender Temperatursenkung bis 36°.8, während die vier übrigen überlebten. Eine Temperatursenkung von 1—3° trat jedoch in allen Fällen ein und hielt noch am zweiten oder sogar dritten Tage an. 4 mg Base in Agar ergab nur eine kurze Senkung (Tabelle III). 2 mg Base subkutan in Ringer hatte keine Wirkung an drei Tieren. Wenn dieselbe Menge in Olivenöl gegeben wurde, trat eine protrahierte schwache Senkung (bis 38°.4) in zwei von vier Fällen ein. Bei subkutaner Darreichung der Base waren somit Temperaturwirkungen nur dann zu beobachten, wenn Olivenöl als Vehikel verwendet wurde.

#### Adrenalinzufuhr in die Zerebrospinalflüssigkeit.

Da eine zentrale Wirkung des Adrenalins auf die Temperaturregulation sich nicht von der Hand weisen lässt, haben wir Versuche mit direkter Zufuhr in die Zerebrospinalflüssigkeit angestellt. In einer Versuchsreihe geschah die Injektion in den dritten Ventrikel. Es wurde durch mehrere Kontrollversuche festgestellt, dass eine Injektion von derselben Menge Ringerlösung in derselben Höhe keine sichere Wirkung ausübte, auch für den Fall, dass die Injektion in die umgebende Hirnsubstanz gelangte, und ferner dass die Narkose an sich nur eine mässige Senkung der Temperatur zur Folge hatte.

Die Wirkung war eindeutig und zeigte sich in einer Temperaturerhöhung, sowohl unter Verwendung des Hydrochlorids als der Base. Schon mit einer Menge von 0.4 mg der Base wurde eine Temperatursteigerung auf  $40^{\circ}.6$  beobachtet, und nach 1 mg bis  $41^{\circ}.3$ . In einem Falle blieb jedoch die Temperatursteigerung aus. Wenn grössere Mengen der Base, z. B. 5 mg intraventrikulär gegeben wurden, waren die Wirkungen mehr stürmisch. Somit trat in 2 von 3 Fällen der Tod des Versuchstieres binnen wenigen Stunden ein, nach vorangegangener Temperatursteigerung, die in einem Fall den extremen Wert von  $43^{\circ}.7$  C. erreichte. Im dritten Fall war der Effekt protrahiert und die Temperatursteigerung weniger deutlich (Tabelle IV—VI).

Auch mit dem Hydrochlorid ergaben sich ähnliche Resultate. Mit 0.5 mg liess sich somit in zwei Fällen eine Temperatursteigerung bis über  $41^{\circ}$  erzeugen. (Tabelle VII.)

Tabelle IV.

15.XI.

16.XI.

7 <sup>45</sup>	14 <sup>00</sup>	16 <sup>15</sup>	21 <sup>00</sup>	7 <sup>30</sup>	13 <sup>00</sup>	18 <sup>30</sup>	Intraventrikuläre Injektion (in 1 ml Ringer)
38.8	39.2	39.9	40.6	39.6	39.1	39.6	15 <sup>40</sup> 0,4 mg Base

Tabelle V.

19.I.

20.I.

16 <sup>35</sup>	17 <sup>00</sup>	17 <sup>30</sup>	17 <sup>50</sup>	22 <sup>15</sup>	9 <sup>30</sup>	13 <sup>15</sup>	Intraventrikuläre Injektion (in 1 ml Ringer)
39.5	37.5		38.9	41.3	39.9	39.6	16 <sup>45</sup> 1 mg Base
		39.5	39.3	40.2	39.4	39.1	17 <sup>30</sup> Kontrolle

Tabelle VI.

12.III

13.III

14 <sup>00</sup>	15 <sup>45</sup>	16 <sup>25</sup>	16 <sup>35</sup>	17 <sup>00</sup>	17 <sup>40</sup>	20 <sup>45</sup>	8 <sup>45</sup>	Intraventrikuläre Injektion (in 0.5 ml Ringer)
39.2	38.5	38.8	39.0	39.2	39.1	39.1	40.1	15 <sup>35</sup> 5 mg Base
39.5	38.8	39.5	39.7	40.8	41.9	43.7	+	16 <sup>00</sup> „
39.3		39.4	39.7	40.1	40.7	+	+	16 <sup>30</sup> „



Tabelle VII.

29.I.

30.I.

10 <sup>00</sup>	11 <sup>30</sup>	12 <sup>10</sup>	12 <sup>35</sup>	13 <sup>05</sup>	13 <sup>45</sup>	20 <sup>10</sup>	9 <sup>00</sup>	Intraventrikuläre Injektion (in 0.5 ml Ringer)
39.0	38.5	38.9	39.9	40.1	40.2	40.0	38.9	11 <sup>35</sup> 0.5 mg Adr. HCl
39.4	39.1	38.7	38.6	39.8	41.1	40.0	39.5	12 <sup>00</sup> 0.5 mg Adr. HCl
39.5		39.7	39.2	38.0	38.4	39.7	39.5	12 <sup>30</sup> Kontrolle
39.8			39.5	39.2	40.3	41.2	40.3	12 <sup>55</sup> 0.5 mg Adr. HCl

Bei Versuchen dieser Art ist selbstverständlich mit der Möglichkeit einer wärmestichartigen Wirkung der Injektion zu rechnen wegen Verletzungen beim Einstich. Gegen eine solche Erklärung der Temperatursteigerung spricht indessen einmal, dass die Kontrollen mit Injektion von Ringerlösung in der Regel ohne Temperaturwirkung waren (in einem Fall wurde 40.2° beobachtet) und zweitens, dass auf feine Nadelstiche selten eine Hyperpyrexie folgt, auch wenn sie in der typischen Gegend der Wärmesticherzeugung treffen (F. BRUMAN, 1941). Die Ergebnisse stehen auch mit denen von anderen Untersuchern (CLOETTA und WASER, 1916, KONDO, 1919) in Einklang.

In weiteren Versuchen wurde geprüft, ob sich auch bei intrazisternaler Injektion Temperatursteigerung erzeugen liess. Dies war tatsächlich der Fall, sowohl bei Verwendung des Hydrochlorids wie der Base. Der Effekt war wie erwartet in hohem Grade von der Dosis abhängig. Ein Beispiel der Wirkung von Injektion einer Suspension von 2 mg Adrenalinbase in 0.5 ml Ringerlösung wird unten in der Fig. 1 a veranschaulicht.

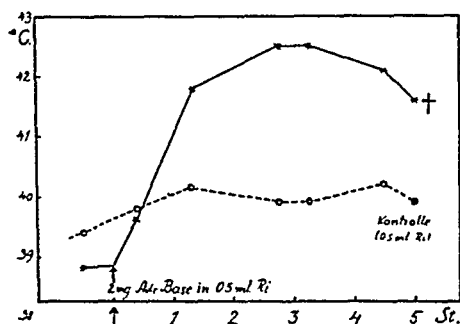


Fig. 1 a. Kaninchen. Rektaltemperatur nach intrazisternalen Injektionen von 2 mg Adrenalinbase in 0.5 ml Ringerlösung, bzw. 0.5 ml Ringerlösung allein (Kontrolle).

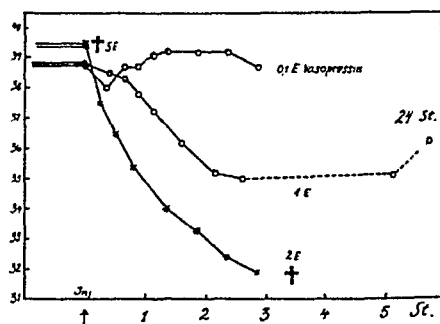


Fig. 1 b. Intrazisternale Injektion von 0.1, 1, 2 und 5 E. Vasopressin. Abszisse: Zeit in Stunden nach der Injektion.

Die Ergebnisse sämtlicher intrazisternalen Injektionen der Base und des Hydrochlorids werden unten mitgeteilt.

Tabelle VIII.

*Intrazisternale Injektion von Adrenalinbase.*

Menge	Rektaltemperatur °C.		
	vor	ca. 2 Stunden nach	16—18 St. nach d. Injektion
0.3 mg . . .	38.8	40.0	39.1
1 „ . . .	37.8	40.4	38.9
1 „ . . .	38.4	41.0	39.0
1—2 „ . . .	39.7	41.8	38.6
2 „ . . .	39.8	41.6	39.2
2 „ . . .	38.8	42.5	†
2 „ . . .	39.4	41.9	38.0
2 „ . . .	39.1	42.8	35.5(†) <sup>1</sup>
2 „ . . .	38.9	43.1	†
2 „ . . .	39.7	42.2	39.3
4 „ . . .	39.6	42.2	34.9

Tabelle IX.

*Intrazisternale Injektion von Adrenalinhydrochlorid.*

Menge	Rektaltemperatur °C.		
	vor	ca. 2 Stunden nach	16—18 St. nach d. Injektion
0.02 mg . . .	39.2	39.7	39.7
0.05 „ . . .	39.2	39.1	39.1
0.05 „ . . .	39.4	39.0	39.0
0.05 „ . . .	39.3	39.6	38.7
0.1 „ . . .	39.1	40.4	38.8
0.1 „ . . .	39.8	41.3	39.8
0.1 „ . . .	38.8	37.4	38.8
0.1 „ . . .	39.1	39.5	39.4
0.2 „ . . .	39.6	37.6	39.7
0.2 „ . . .	38.9	40.3	39.8
0.3 „ . . .	39.5	37.3	35.3
0.5 „ . . .	39.1	37.8	35.7

<sup>1</sup> Starb am folgenden Tag.

Aus der Tabelle VIII ist ersichtlich, dass die Wirkung der Adrenalinbase auf die Körpertemperatur des Kaninchens sehr ausgesprochen sein kann. Mit Dosen unter 1 mg ist die Wirkung unsicher, aber schon mit 1 und noch mehr mit 2 mg wird eine mächtige Steigerung beobachtet. Das Maximum tritt nach etwa 2 Stunden ein und nachher fällt die Temperatur allmählich ab. In vier Fällen sank die Temperatur unter den Anfangswert und in drei Fällen ging das Tier ein. Mit 4 mg war die postpyretische Temperatursenkung sehr ausgesprochen und hielt noch 3 Tage an. In sämtlichen Fällen zeigten die Tiere mit Temperatursteigerung eine ausgesprochene Polypnoe sowie motorische Unruhe, wie auch frühere Untersucher beobachtet haben (HASHIMOTO, 1915). Die temperatursteigernde Wirkung des Hydrochlorides war, wie die Tabelle IX zeigt, weniger deutlich. Nur in einem Fall (nach 0.1 mg) überstieg die Temperatur  $41^{\circ}$ . Andererseits wurden mit Dosen von 0.1 mg an sichere Temperatursenkungen beobachtet, die nach 0.3—0.5 mg sehr ausgesprochen waren.

Da die Base in Ringer als eine Suspension vorliegt, haben wir entsprechende Suspensionen von Kaolin in Ringerlösung injiziert. Die Temperatureffekte waren hierbei sehr unbedeutend und deckten sich etwa mit denen von alleiniger Ringerinjektion. Wenn grössere Kaolinmengen injiziert wurden, (etwa 50 mg) trat in einem Fall eine beträchtliche Senkung der Temperatur ein.

Es schien uns ferner von Interesse zu untersuchen, in welchem Masse die Suspension von Adrenalinbase gelöstes Adrenalin liefert. Die Suspension wurde mit menschlicher Zerebrospinalflüssigkeit oder bikarbonatfreier Ringerlösung bereitet, (2 mg in 1 ml), und bei  $38^{\circ}$  gehalten. Der Adrenalingehalt der sich bald rot bis gelb färbenden Lösung (pH 7) wurde gegen Adrenalinhydrochlorid als Standard auf Kaninchenbarm oder Katzenblutdruck biologisch geprüft. Die gefundenen Werte werden unten mitgeteilt (Tabelle VIII).

Tabelle X.

Z e i t	Gelöstes	Adrenalin, mg	pro ml	(aus 2 mg).
5' . . . . .	0.06			
15' . . . . .	0.38			
30' . . . . .		0.3		
45' . . . . .	0.63	0.6		0.5
90' . . . . .		0.8		0.75
120' . . . . .	0.98	1.0	1.25	

Die erste Spalte bezieht sich auf einen Versuch, wo die Lösung vor jeder Prüfung abzentrifugiert wurde und eine neue Quantität Lösungsmittel hinzugesetzt wurde. Die Zahlen stellen die summierten Werte dar und sind mit denjenigen in den folgenden Spalten direkt vergleichbar, wo der Gehalt in der Lösung zu verschiedenen Zeiten bestimmt wurde.

Aus den Versuchen geht hervor, dass die Adrenalinbase fortlaufend Adrenalin in Lösung abgibt. Erneute Prüfung der abzentrifugierten Lösungen am folgenden Tage zeigte keine merkliche Abschwächung.

### **Intrazisternale Injektionen von Vasopressin.**

Da die starke temperatursenkende Wirkung grösserer Adrenalin-dosen einen vasomotorisch bedingten Effekt vermuten lässt, (cerebrale Ischämie) haben wir einige Versuche mit dem gefässverengenden Stoff des Hypophysenhinterlappens angestellt. Die Ergebnisse waren ganz eindeutig und zeigten bei intrazisternaler Darreichung von 1 E. eine Temperatursenkung bis 35° und nach 2 E. bis unter 32° (Fig. 1 b). Bei der letzterwähnten Dosis starb das Versuchstier nach 3 Stunden. 5 E. Vasopressin führten den Tod des Tieres fast unmittelbar herbei. Mit einer Dosis von 0.1 E. war die Temperaturwirkung unsicher. Eine Temperatursenkung nach Hypophysenhinterlappenextrakt fanden schon JACOB und RÖMER (1912). Diese Ergebnisse stützen die Ansicht, dass höhere Dosen von gefässverengenden Stoffen eine zerebrale Ischämie verursachen, die mit einer allgemeinen Lähmung zerebraler Funktionen einhergeht.

### **Ergotaminhemmung der Adrenalinwirkung bei intrazisternaler Injektion.**

Eine Aufhebung der temperatursteigernden Wirkung von intrazisternal zugeführtem Adrenalin konnte mit Ergotamin (Gynergen Sandoz) erzeugt werden, wobei die letzterwähnte Substanz ebenfalls intrazisternal gegeben wurde.

Mit 0.1 mg Ergotamin und 2 mg Adrenalinbase stieg die Temperatur bis 42.1°, also etwa auf die gewöhnliche Höhe. Nach 0.25 mg Ergotamin andererseits wurde keine Steigerung beobachtet sondern nur eine Senkung, die vom Tod des Versuchstieres gefolgt war. Eine ähnliche antagonistische Wirkung von Ergotoxin auf

das Adrenalinfieber ist von DÖBLIN und FLEISCHMANN (1913) beobachtet worden. RIGLER und SILBERSTERN (1927) fanden, dass Ergotamin und Ergotoxin an sich die Körpertemperatur herabsetzten, besonders bei subduraler Darreichung.

### Besprechung.

In Übereinstimmung mit früheren Untersuchern haben wir bei subcutaner Zufuhr von Adrenalin in kleinen Dosen in einzelnen Fällen eine leichte Temperatursteigerung des Kaninchens beobachtet. Grössere Mengen ergaben regelmässig eine Temperatursenkung. Wurde statt dessen das Adrenalin in die Zerebrospinalflüssigkeit injiziert, trat mit geeigneten Dosen eine viel stärkere Temperatursteigerung ein, die 43.°7 C. erreichen konnte. Besonders nach Injektion einer Suspension der Base trat eine beträchtliche Hyperpyrexie ein, die mit Adrenalin früher nicht beobachtet zu sein scheint. Der Grund für die stärkere Wirkung bei Zufuhr der Base dürfte darin zu suchen sein, dass hierbei eine gewisse Depotwirkung zur Auswirkung gelangt, d. h. kleine Mengen werden fortlaufend abgegeben, etwa wie man sich bei erhöhter Abgabe im Körper denken könnte. Der Hauptangriffsort der Adrenalinwirkung ist zweifelsohne zentral, wobei es zunächst unentschieden bleibt, ob die Wirkung über die Gefässe, oder direkt an den nervösen Elementen erfolgt. Besonders bei den höheren, temperatursenkenden Dosen dürfte eine Gefässwirkung (Kontraktion — Ischämie) mit in Rechnung bezogen werden, was durch die Versuche mit Hypophysenhinterlappenhormon gestützt wird. Die Adrenalinapnoe (ROBERTS, 1910) und sekundäre Blutdrucksenkung an Kaninchen (VAN LEERSUM, 1909, EULER, 1938 a) sprechen in derselben Richtung.

Für das Zustandekommen der Temperatursteigerung ist wie bei anderen Fieberzuständen mit einer gestörten Wärmerregulation zu rechnen. Die erhöhte vasomotorische Aktivität (Kontraktion der Ohrgefässe) sowie die motorische Unruhe sind den Symptomen des »Schüttelfrostes« bei infektiösen und anderen Fieberzuständen ähnlich. Über die Beziehungen zwischen Hyperadrenalinämie und Fieber (EULER, 1927) haben unsere Versuche insofern Aufschluss geliefert, als sie zeigen konnten, dass die erste wahrscheinlich nicht für eine Hyperpyrexie verantwortlich sein kann, da bei extrazerebraler Zufuhr von Adrenalin nur mässige Temperatursteigerungen im Vergleich mit denjenigen bei

zentraler Applikation beobachtet wurden. Pyretisch wirksame Stoffe können anderseits einen Sympathicusreiz und Adrenalin-ausschwemmung bewirken.

Der Angriffsort des Adrenalins im Gehirn ist nicht bekannt. Wie bei den sehr ausgesprochenen Wirkungen kleiner K-mengen intrazisternal, (EULER, 1938 b) dürfte für das Adrenalin gelten, dass das reaktive Gewebe ziemlich oberflächlich in der Ventrikelwand des Gehirns liegt. Was den Wirkungsmechanismus des Adrenalins anbetrifft, wenn man eine Aktivierung bestimmter nervöser Strukturen annimmt, so ist man wenig unterrichtet. Adrenalin scheint nicht wie z. B. Azetylcholin allgemein synaptotrop zu wirken, obwohl gewisse Beobachtungen für eine bedeutende Rolle des Adrenalins bei der Überführung von neuromuskulären Erregungen und von der Übertragung im Neuron selbst sprechen (BJURSTEDT und EULER, 1939, BÜLBRING und BURN, 1939). Nach HAAS (1939) vermindert Adrenalin bei intrazisternaler Injektion den Acetylcholingehalt des Hirnstammes beim Kaninchen, weshalb auf eine zentrale Wirkung geschlossen wird.

Eine interessante Wirkung an der Netzhaut hat THERMAN beschrieben (1934), indem er u. a. eine starke Aktivierung der c-Welle des Elektroretinogramm nach Adrenalin beobachtete.

### Zusammenfassung.

Intraventrikuläre oder intrazisternale Injektion von Adrenalinhydrochlorid an Kaninchen erhöht die Körpertemperatur in Dosen von 0.1—0.2 mg mit 1—2° C. Bei Verwendung einer Suspension der Base in Ringerlösung in Dosen von etwa 2 mg traten viel stärkere Temperatursteigerungen auf (bis 43°.7).

Eine hochgradige Temperatursenkung erfolgte mit grösseren Adrenalindosen und trat ebenfalls mit Vasopressin ein, was als Folge zerebraler Ischämie und Lähmung gedeutet wird.

Ergotamin verhinderte bei intrazisternaler Darreichung in Dosen von 0.12 mg pro kg die temperatursteigernde Wirkung des Adrenalins.

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## Contributions to the Chemistry of Prothrombin.

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Different methods for preparation of prothrombin-containing solutions are known (cf. the review by WÖHLISCH (1940)), but potent preparations have been made only by J. MELLANBY (1930) and SEEGER, SMITH, WARNER and BRINKHOUS (1938). Now we have tried to convert our method for preparation of thrombin (ASTRUP and DARLING (1940, 1941)) into a method for preparation of prothrombin.

As a unit for prothrombin (P. T. U.) we have chosen the amount of prothrombin which yields one unit of thrombin (T. U.), when fully converted into thrombin by the addition of thrombokinase (thromboplastin) and calcium ions. The potency of thrombin is defined and its measurement carried out as described recently by ASTRUP and DARLING (1942 b).

### Preparation of Prothrombin.

The prothrombin solution is made from oxalated plasma according to the method of MELLANBY (1930) as described in our preparation of thrombin (ASTRUP and DARLING (1940, 1941)). The prothrombin-containing MELLANBY fibrinogen resulting from 10 liters of plasma is dissolved at neutral reaction in 1,500 ml of physiological NaCl heated to 37° and containing 0.3 per cent of potassium oxalate. From the solution obtained fibrinogen is removed after MELLANBY (1909) by adding a small amount of a thrombin solution (about 1,500 T. U. in 50 ml)



After removal of the fibrin formed a solution containing from 8,000 to 20,000 P. T. U. per liter is obtained. This solution,  $P_0$ , is very unstable and loses its prothrombin content in two to six hours. This property appears to be due to proteases present in the solution (see below).

The solution  $P_0$  is diluted with 15 volumes of distilled water and one per cent acetic acid added to a pH of 5.30 (about 50 ml). The precipitate is removed by centrifugation and treated several times with acetone. After treatment with ether it is dried in the air. Yield: 20–35 g of a white powder,  $P_1$ , containing from 40 to 60 per cent of the prothrombin present in  $P_0$ .

While the activity of  $P_0$  was about 85 P. T. U. per mg of nitrogen, the activity of  $P_1$  is about 175 P. T. U. per mg of N, and one g of dry powder contains about 1,600 P. T. U.

For measurement of the potency 1.0 ml of a prothrombin solution is activated by adding 0.1 ml of a thrombokinase prepared from ox lung as described earlier (ASTRUP and DARLING (1942 a)) or, most often, with a kinase prepared by making a suspension of ox brain (as will be described in a subsequent paper). After further addition of 0.1 ml of 1.5 per cent  $\text{CaCl}_2$ , sicc. the mixture is placed in a water bath at 37°. The course of the activation process is followed

by determining as usual the thrombin formed. As a rule the prothrombin has undergone total activation in about 10 to 30 minutes. For the measurement samples on 0.10 ml of the solution, which is undergoing activation, are used. The amount of units found must be multiplied by 12 to give the amount of P. T. U. in one ml of the original prothrombin solution. Fig. 1 shows two typical examples of the activation process. Solutions of  $P_1$  are used.

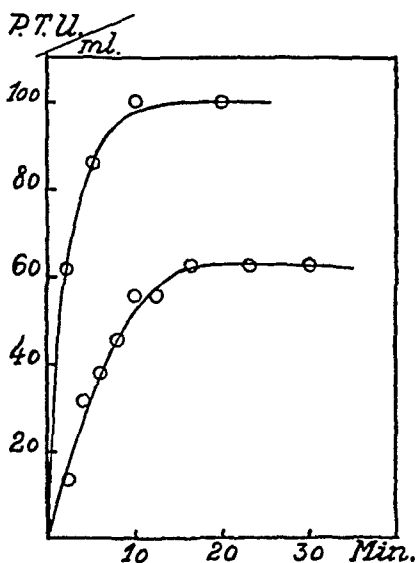


Fig. 1. Activation of prothrombin by addition of thrombokinase and  $\text{Ca}$  ions.

### Properties of the Prothrombin $P_1$ .

The properties of  $P_1$  were investigated in order to find a method for purification, but although several different methods were tried, it has not yet been possible to devise a satisfactory purification method. Prothrombin seems to be more unstable than thrombin, and especially it has been impossible to remove the accompanying proteases from the prothrombin solutions.

At first different solutions for extraction of the prothrombin from the dry  $P_1$  were tried. 0.5 g was treated with 15 ml of the solvent for one hour and the potency of the resulting extract measured after adjusting the pH to 7.0. The results are recorded in Table 1. If necessary the solution is added NaOH until neutral reaction.

Table 1.

Solvent	P. T. U. per ml
Physiol. NaCl . . . . .	22
1-m NaCl . . . . .	0
0.1-m NaAc . . . . .	22
0.33-m NaAc . . . . .	33
1-m NaAc . . . . .	43
1 per cent pyridine . . . . .	16

According to this result all the subsequent measurements and experiments were carried out with 1-m sodium acetate as solvent.

On examination of the precipitability of  $P_0$  and  $P_1$  with addition of ammonium sulphate according to SCHMITZ (1933) the curves shown in Fig. 2 were obtained.

From these curves it is seen that both  $P_0$  and  $P_1$  show a considerable content of globulin but only small amounts of albumin. Prothrombin therefore seems to be a globulin, while it has already been pointed out (ASTRUP and DARLING (1940, 1941)) that thrombin is an albumin. From the curves it is also seen that further purification by precipitation with ammonium sulphate does not seem

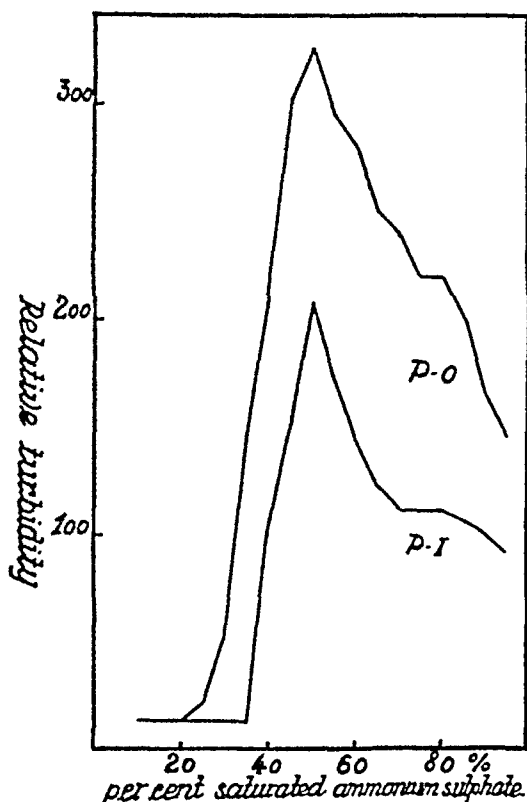


Fig. 2. Ammonium sulphate precipitation curves of prothrombin solutions.

promising, which is contrary to the purification of thrombin, where it was possible in this manner to remove inactive globulins from the active albumin fraction. The conversion of prothrombin into thrombin by means of thrombokinase and calcium ions therefore seems to be a formation of an albumin from a globulin. It has been mentioned previously (ASTRUP (1941 a)) that thrombin seems to be a protein of rather low molecular weight as in contrast to prothrombin it passes slowly through cellophane membranes. It does not contain phosphorus and cannot therefore consist of a combination between prothrombin and the phosphorus-containing thrombokinase. It seems more likely that a globulin, prothrombin, is converted to a smaller albumin molecule, *i. e.* thrombin, by the action of thrombokinase and calcium ions. The formation of thrombin may probably be looked upon as a proteolytic process, which is very well in accordance with the activation of prothrombin into thrombin by means of trypsin (EAGLE (1937), FERGUSON and ERICKSON (1939)).

Recently we have succeeded in demonstrating this transformation directly by making use of ammonium sulphate precipitation curves of prothrombin solutions before and after activation with thrombokinase and calcium ions (ASTRUP and DARLING (1942 c)). Both by using thrombokinase prepared from ox brain and from ox lung it was possible to show the presence of a small amount of albumin after the activation, while the solution without addition of thrombokinase showed only globulin character. In order definitely to settle this question it will be necessary, however, to obtain still more purified prothrombin solutions, as only a small fraction of the globulins in the present preparations consists of pure prothrombin and can be transformed into thrombin.

For this purpose different methods were tried, especially such adsorption processes as described by SEEGERs, SMITH, WARNER & BRINKHOUS (1938), but in no case was it possible to exclude the contamination with proteases which inactivate the prothrombin solutions in the course of a few hours and therefore make the handling and investigation of the solutions very difficult. It was therefore found necessary to postpone further purification experiments until it was found possible to prepare better starting materials than solutions prepared after our thrombin method. The better results with the purification of thrombin may be due to its properties as an albumin. The original methods of MELLANBY (1930) and SEEGERs, SMITH, WARNER & BRINKHOUS (1938) are inconvenient for practical purposes when larger amounts are wanted, but they seem to give more stable and easily purified products. In a solu-

tion of MELLANBY fibrinogen prepared in the same manner from *chicken* plasma, the prothrombin is much more stable than that obtained by using *ox* plasma (ASTRUP (1941 b)).

However, the Iowa workers themselves have found an inactivation of prothrombin solutions (MERTZ, SEEGERs and SMITH (1939)), but only in the presence of thrombin, and they are of the opinion that thrombin itself is the substance responsible for the inactivation. It may be due, however, to activation of proteases, as it is well known that proteases are present in plasma in inactive form. They may probably be activated in the same manner as prothrombin and are therefore found in the thrombin solutions. HUDEMANN (1940) has shown that it is possible to differentiate between the clotting and the fibrinolytic action of thrombin preparations, and that the last-mentioned property must be due to contamination with a proteolytic enzyme. Further, FERGUSON (1941) has shown that it is possible to prepare purified solutions of prothrombin and thrombin which are stable when mixed. He also is of the opinion that the trouble is caused by proteases. COLLINGWOOD and MAC MAHON (1913) seem to have been the first to observe a deterioration of prothrombin and they postulate the presence of an "antiprothrombin" in plasma which must not be confused with heparin, the antiprothrombin of HOWELL. They demonstrate it in MELLANBY-precipitated prothrombin after removal of fibrinogen by means of thrombin, *i. e.* just in the same manner as in our experiments. The antiprothrombic action in their case therefore seems to be due to the same substances as those here in question.

By heating to 56° for 10 minutes at neutral reaction the prothrombin is almost totally destroyed (cf. SEEGERs (1940)).

### Summary.

1. A unit for prothrombin (P. T. U.) is defined as the amount of prothrombin which by total activation yields one unit of thrombin.
2. Prothrombin is prepared from *ox* plasma. It seems to be a protein of globulin character.
3. The transformation of prothrombin into thrombin seems to be a conversion of a globulin into an albumin.

This work was aided by grants from "Danmarks tekniske Højskoles Fond for teknisk Kemi" and from "Løvens kemiske Fabrik" Copenhagen.



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## A Note on the Cis-trans Specificity of Liver Esterase.

By

GUNNAR STEENSHOLT.

Received 1 October 1942.

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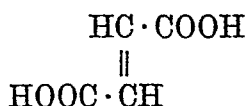
The problem of specificity is of major importance in the investigation of any enzyme. This is due to the fact that the specificity properties of an enzyme may be expected to afford a valuable clue to the understanding of its chemical nature and biological function. A very large amount of experimental work has consequently been done on this problem for practically every known enzyme, the substrates of which exhibit the property of isomerism. The interest has thereby been confined almost exclusively to optical specificity, which is probably also the most important specificity property met with so far in enzyme chemistry. However, for certain enzymes the question of the existence of other types of specificity may arise. This is especially the case for the esterases, and in particular the lipases, where a large number of possible substrates exist, which possess the property of cis-trans isomerism. The problem is, then, whether the esterases show a corresponding cis-trans specificity.

The question of the existence of a cis-trans specificity for esterases has already been raised in the literature (NORD and WEIDENHAGEN, 1939). Beyond this, however, it does not seem to have received much attention, and a complete answer to the question is nowhere to be found. There is a very interesting paper by FABISCH (1931), in which, among other things, the esterification of fumaric and maleic acid under the action of pancreas lipase is investigated. The discussion is, however, exclusively confined to the synthesising properties of the enzyme. MCGINTY and LEWIS (1926) in the course of their work on the enzymic hydrolysis of esters of dicarbonic acids also more incidentally investigated the

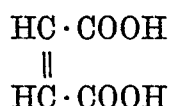
ethyl esters of fumaric and maleic acids; this is the only reference to experimental work on the problem which we have been able to find. These authors find that ethyl fumarate is hydrolyzed considerably more rapidly than ethyl maleate; the latter substrate is attacked by the enzyme only with a certain difficulty. They worked with glycerol extracts of the liver of the pig, and determined the hydrolysis titrimetrically by adding alkali after the lapse of, say,  $\frac{1}{2}$ , 1,  $\frac{3}{2}$ , . . . hours. This procedure suffers from a rather serious drawback, namely the greatly changing hydrogen ion concentration in the reaction mixture. This circumstance may strongly influence the result, and it is therefore necessary to reinvestigate the problem with methods which do not suffer from this defect.

The present work was begun and carried through independently of that of MCGINTY and LEWIS, whose paper did not come into our hands until after the measurements were completed. To follow conveniently the progress of the hydrolysis the well known titrimetrical method of KNAFFL-LENZ (1923) was applied, which is very simple and convenient and, when properly carried out, ensures a fairly constant hydrogen ion concentration during the experiment. However, the choice of indicator is, in view of the work of BAMANN and SCHMELLER (1931), a matter of great importance. In a careful investigation these authors have shown that several commonly used indicators exert a more or less strongly restraining influence on the enzymic process, and at sufficiently high concentrations their use may lead to completely erroneous results. To avoid this difficulty one has to work with as low concentrations of the indicator dye as possible and, of course, to choose the right dye. There are only a few indicators available which are suitable for use in our particular problem. Fortunately, BAMANN and SCHMELLER succeeded in showing that brom cresol purple and neutral red are without any perceptible influence on the enzymic hydrolysis, at any rate as long as they are present in sufficiently low concentrations, and these maximal concentrations were shown to be high enough to allow of good colour reactions. These indicators can therefore be used with confidence, and they have consequently been applied in the present series of measurements. However, in some experiments phenolphthalein and brom thymol blue were also used, in order to show that the qualitative nature of the results is independent of the particular indicator used, and of the hydrogen ion concentration of the reaction mixture (at any rate for the alkaline range).

The substrates used were the diethyl esters of fumaric and maleic acids:

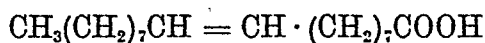


Fumaric acid (trans form)

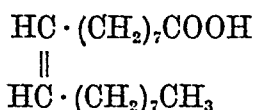


Maleic acid (cis form)

and the ethyl esters of elaidic and oleic acids:



Elaidic acid (trans form)



Oleic acid (cis form)

The general results obtained may be briefly summarized in the statement that ethyl fumarate is hydrolysed considerably faster than ethyl maleate. For the other two geometrical isomers the velocity of the enzymic process is much smaller and no noticeable difference between them was observed with certainty. In other words the effect is very pronounced in the former case and practically absent in the latter (the differences observed being here within the limits of error).

The exact interpretation and explanation of these findings are at present necessarily somewhat uncertain, in the absence of any deeper insight into the mechanism of enzymic hydrolysis and the chemical constitution of the esterases. Briefly the results may perhaps be expressed by saying that in the trans form (ethyl fumarate) both ester groups are attacked more or less simultaneously, while in the cis form (ethyl maleate) a simultaneous attack on the ester groups is made difficult or even impossible. This may again be linked up with the electrochemical nature of the carboxyl and ester groups, and their mutual interaction, which is certainly different in the trans and the cis form (BAMANN and RENDLER 1935). In the other example of geometrical isomerism investigated here, no such mutual interaction between carboxyl and ester groups takes place, and the position (cis or trans) of the more or less electrically neutral  $\text{CH}_3(\text{CH}_2)_7 \cdot \text{CH}$ -group relative to the carboxyl or ester group may be assumed to be rather irrelevant. It should be stressed, however, that attempts of this kind at a theoretical explanation are, in view of what has just been said about the incompleteness of our present knowledge, of a very tentative nature.



## Experimental part.

*Substrates.* Ethyl fumarate and ethyl maleate were synthesised according to the method described in Organic Synthesis 10, 48. The esters were purified by redistillation. The same method was applied to the ethyl esters of oleic and elaidic acid; in this latter case the distillations were carried out under diminished pressure.

*Enzyme solution.* The measurements were carried out using a glycerol extract from pig's liver. As is well known these extracts have the advantage of a low protein content, and when properly stored in the ice-box they retain their activity for a considerable time. A part of the liver was cut into pieces and forced twice through a meat mill. It was then carefully ground with sea sand, after which it was extracted for several hours with glycerol and finally filtered carefully.

The KNAFFL-LENZ method is so well known that we can safely refrain from a closer description of it. In all cases here recorded the experiments were carried out with 0.3 g of the ester suspended or emulgated by thoroughly shaking it with 100 cm<sup>3</sup> of water. The enzyme solution and the indicator were then added, and the continuous titrations carried out with *n*/10 NaOH or *n*/40 NaOH. To ensure comparable conditions the same amount of indicator and the same amount of enzyme solution were used in each measurement. As regards the dosage of the indicators care was taken to utilize the results of BAMANN and SCHMELLER discussed above in order to minimize as much as possible the influence of the dyes on the enzymic process. Furthermore, in the tables given below, the values measured have been corrected for the self-hydrolysis of the esters, which was determined in separate experiments. All measurements were carried out at a temperature of  $20^{\circ} \pm 0.1$  C.

Table I contains the results for ethyl fumarate and ethyl maleate, and Table II the results for the ethyl esters of elaidic and oleic acids.

Each measurement was performed twice, the results differing from each other by not more than 5—6 percent.

Experiments were also carried out over both shorter and longer periods of time and with varying amounts of ester and enzyme solution. We refrain from reproducing all these measurements here, and confine ourselves to the above results, which are typical.

Table I.

$\text{Cm}^3 \frac{n}{10} \text{NaOH}$  added after  $1\frac{1}{2}$  hour.

Indication	Brom cresol purple	Phenol phtalein	Brom thymol Blue	Neutral red
Ethyl fumarate .	4.5	11.4	9.1	9.4
Ethyl maleate .	3.9	8.4	7.4	7.4

Table II.

$\text{Cm}^3 \frac{n}{40} \text{NaOH}$  added after 1 hour.

Indication	Brom cresol purple	Phenol phtalein	Brom thymol Blue	Neutral red
Ethyl elaidate . .	3.1	5.9	4.0	2.9
Ethyl oleate . .	3.0	5.7	4.0	2.8

The author expresses his best thanks to Prof. E. BILMANN and Prof. R. EGE for their hospitality. It is also a pleasure for him to express his thanks to Lektor ST. VEIBEL for the interest he has taken in the present work and for several discussions.

### Summary.

An investigation is made of the hydrolysis by liver esterase of the ethyl esters of fumaric and maleic acid, and of oleic and elaidic acid. It is found that in the former case the reaction velocity is considerably higher for ethyl fumarate than for ethyl maleate. In the latter case no appreciable difference in reaction velocity is found. The possible interpretation of the phenomenon is briefly discussed.

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Stockholm.

## **"Red" and "Green" Receptors in the Retina of *Tropidonotus*.**

By

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Received 3 October 1942.

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In our survey of colour receptors in the retina, carried out with micro-electrodes stuck into the opened eye, frogs, rats, pigeons, guinea pigs, the Greek tortoise and a number of fishes have been studied (GRANIT and SVAETICHIN, 1939, GRANIT 1941—42). In this paper the work is extended to a snake (*Tropidonotus natrix*) which is of interest because of its pure cone retina (see *e. g.* WALLS, 1934).

The technique for energy control of the spectrum and application of the micro-electrode has been described in the earlier papers of this series (*cf.* in particular GRANIT and SVAETICHIN, 1939, and GRANIT 1941, a) and need not be redescribed. The snakes were anaesthetized with urethane (20 %) and the micro-electrode inserted with micro-manipulator into the opened eye under suitable magnification of the visual field. Successful experiments were carried out with 11 eyes from which 18 series were obtained, a series referring to a given location of the micro-electrode and comprising a large enough number of observations for establishing the spectral locus of its sensitivity curve. The number of values (threshold determinations) obtained in such a series may vary from 5 to 50, each figure being fixed upon after several positive and negative readings on both sides of the threshold. The values are averaged and plotted in per cent of the inverse value of the energy necessary for a threshold response in each wave-length tested.

### **General Observations.**

Spikes from single elements or from elements synchronized to act as singles are sometimes obtained, though not as often and easily as in mammals. Relatively restricted discharges are the rule.

The slow respiration may cause variations in the level of sensitivity which have to be followed by continuously calibrating with the wavelength chosen as control — nearly always the maximum.

In animals with rods and some type of visual purple nearly all receptors later enter upon a secondary phase of dark adaptation pushing the region of maximal sensitivity towards the spectral locus of the top of the absorption curve for visual purple. This always means a shift of the sensitivity towards the short wavelengths. It also means that the spectral properties of elements located by the microelectrode have to be analysed before dark-adaptation sets in. In the cone-retina of the snake this complication is absent and there is no need for light-adapting the animals. To be true, the green region of the spectrum has sometimes become more prominent later in an experiment when the animal has been lying in the dark box and its eye only illuminated by threshold stimuli. But this need not mean more than that the “green” elements are more sensitive to the light adaptation necessary for inserting the micro-electrode, and the change is not of the order of magnitude characterizing dark-adaptation in eyes containing visual purple. The pure cone eye of the Greek tortoise behaved in a similar fashion (GRANIT, 1941 b).

In the retina of the snake low intensities merely elicit responses to illumination, no off-discharges. To stronger stimuli, however, most elements react with both on- and off-responses. Pure off-elements have not been found, which, of course, need but mean that they are rare.

The bulb is very soft and small in this animal and consequently easily damaged when opened. Urethane has to be given cautiously in repeated doses of 0.2 cc to ensure optimal conditions. Neither the isolated eye nor the eye of a snake, which does not breathe after urethane, responds to light. Several animals were lost owing to the difficulties mentioned.

### The Colour Receptors.

Elsewhere I have pointed out (GRANIT, 1941, e, f) that the animals with good colour vision possess one broad dominator band and some narrow modulator bands in other regions of the spectrum. The dominator is assumed to represent “brightness” or “white” modulated to “colour” by the modulators. The spectral properties of the visual receptors of *Tropidonotus* conform to this scheme.

The broad dominator band is plotted in fig. 1 from 5 series, one of them referring to an isolated spike. The maximum of the dominator is in  $0.560 \mu$  and to all appearance the curve is identical with the dominator found in, for instance, frogs (GRANIT, 1941, f) and cats (GRANIT, unpublished) and with the luminosity curve of the photopic human eye. In the pigeon the dominator appeared shifted somewhat to the right, probably owing to selective absorption by the coloured oil globules (GRANIT, 1942, b).

Fig. 2 shows 7 series with maximum in  $0.600 \mu$ . All except 2 had a secondary hump in the green though the level of this hump

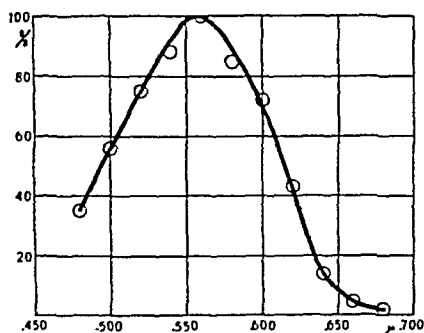


Fig. 1. Average spectral distribution of sensitivity of 150 values from 5 series with "dominator" elements. Spectrum of equal quantum intensity.

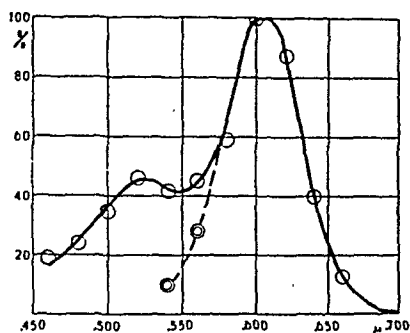


Fig. 2. Average spectral distribution of sensitivity of 124 values from 7 series with well marked "red" modulator and moderate or small secondary hump in the green. Dotted line refers to 2 cases in which hump in the green absent or small. Spectrum of equal quantum intensity.

varied from case to case. In the two remaining series the curve passed down towards  $0.540 \mu$ , as shown by the broken line of the figure, and the threshold of these two elements was so high as to exclude continuation of the experiment farther towards the short wave-lengths. Thus we do not know whether or not a small hump in the green would have appeared in these cases too. However it is clear that the "red" element of fig. 2 with maximum around  $0.600 \mu$  is of the narrow modulator type.

Fig. 3 illustrates 2 series in which the "green" element, coupled to the "red" one, is still more prominent than in fig. 2. It is conceivable that, by combining in a certain proportion, the "red" and "green" modulators may add up to the distribution curve for the dominator. A step in this direction is indicated by the series illustrated by the open circles in fig. 4. The curve drawn through

these observations, (as many as 37) comes very near the dominator. The red is emphasized by a hump around  $0.600 \mu$ . This hump is still more marked in the second curve of the same figure, illustrating another series based on 19 observations, most of which fell around the maxima. The values for  $0.520$  and  $0.540 \mu$  are single values only, taken at the end of the experiment, and may therefore be too low.

A process of fusion of the two modulators into a dominator would certainly provide an attractive explanation of the facts that the dominator is broad and symmetrically placed with respect to the two narrow modulators and that the experiments prove them to be combinable in different proportions. It

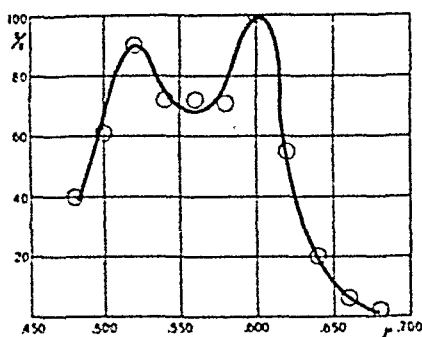


Fig. 3. As fig. 2, but with large rise in the green. Averages of 73 values from 2 series.

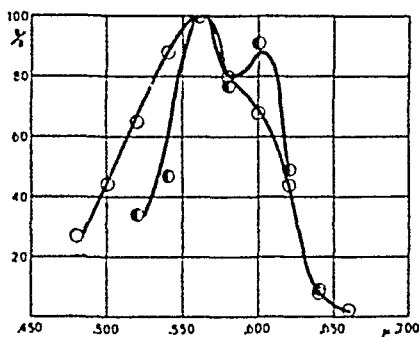


Fig. 4. As fig. 1, but dominator with hump in the red. Averages of 56 values from 2 series.

would also explain that in the animals taken in the spring the dominator was rare and the "red" modulator common whereas in those taken in the autumn the dominator was common and the modulator rare. The "green" modulator was never obtained in the isolated state but that it also is narrow is clearly indicated by the results. Isolated elements were found with all types of curves.

Knowing from the work of POLYAK (1936) that both rods and cones and (or) several cones converge towards the same ganglion cell and that the micro-electrode picks up the discharge from this layer, we can understand results such as those of figs 1—4 by glancing at the diagram of fig. 5. The convergence of "red" and "green" elements towards the same final common path is there illustrated schematically. The different proportions of "red: green" may be thought to represent the experimentally determined curves of figs. 1—3.

If there be a "blue" modulator in the eye of the snake it must be represented by relatively few receptors or by very insensitive ones. It has not been seen in these experiments.

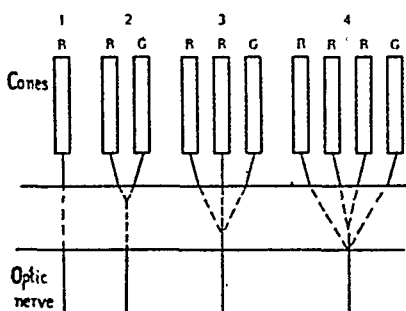


Fig. 5. Diagram illustrating convergence towards optic nerve of "red" and "green" cones in different proportions suggesting an explanation of the curves of figs. 1-4 in terms of two elementary modulators. R = "red" cone, G = "green" cone. 1, "red" modulator alone as in fig. 2 dotted curve; 2, "red" and "green" combining to dominator of fig. 1; 3, two "red" and one "green" modulator combine to give curve of fig. 3; 4, three "red" and one "green" modulator combine to give curve drawn in full of fig. 2.

1941, c) around  $0.520-0.560 \mu$ . The eyes of snakes like those of rats sometimes tend to bleed a little when opened.

The eye of *Tropidonotus* has been used by v. STUDNITZ (1940) for extraction of his so-called "Zapfensubstanz". In his experiments extracts were made on whole bulbs(!) and extinction analyzed with the "Stufenphotometer" of PULFRICH. A very broad composite curve was obtained with maxima — according to v. STUDNITZ — in  $0.468$ ,  $0.555$  and  $0.655 \mu$ . They seem to bear no clear relation to the data obtained with the far more accurate electrophysiological method which, in addition, registers what actually is being led up to the brain.

### Summary.

Micro-electrode, amplifier and cathode ray oscillograph have been used for the recording of "spikes" from restricted units in the retina of anaesthetized snakes in response to monochromatic light of known energy content. The problem has been to study the

The "red" modulator at  $0.600 \mu$  seems to be very constant in different animals. The curve of fig. 2 could just as well have been taken from a frog or a rat. The "green" modulators vary in location from  $0.500-0.530 \mu$  in different animals. On account of the visual purple, which in animals with mixed retinæ is a source of complication in the green region of the spectrum, modulators in this region may often be very difficult to measure with precision. Again, the steepness of the "red" modulator towards the left side of the spectrum may be exaggerated by the filtering effect of haemoglobin (GRANIT,

distribution of sensitivity to monochromatic light of more or less restricted discharges in this cone eye.

There is a broad dominator band of sensitivity with maximum in  $0.560 \mu$ , similar to the dominator found in the frog's eye.

There are further two narrow modulator bands of sensitivity with maxima in respectively  $0.600$  and  $0.520 \mu$ .

The "red" modulator is sometimes obtained in the practically isolated state but generally the "green" modulator appears coupled to it so that a secondary hump of variable size is found with maximum in  $0.520 \mu$ .

The author is indebted to the Rockefeller Foundation for a grant to this laboratory.

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# **Untersuchungen über die Wirkung von antiperniciösen Mitteln, von einer Reihe von Vitaminen und von Eisen auf die Permeabilität der roten Blutkörperchen des normalen Menschen.**

Von

**SØREN L. ØRSKOV.**

Eingereicht am 10. Oktober 1942.

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In einer früheren Arbeit (ØRSKOV 1941 b) konnte gezeigt werden, dass perorale Eingabe von Leberextrakt und Ventriculin eine wesentliche Verlangsamung der Permeierungsgeschwindigkeit von Glukose durch die Membran der roten Blutkörperchen bedingen. Die Wirkung erreicht in den meisten Fällen nach Ablauf eines Tages ihr Maximum und bleibt nach einer einzelnen Eingabe mehrere Tage lang bestehen. Wenn die Einnahme mehrerer Tage lang erfolgt, kann die Wirkung sich über 14 Tage lang halten.

Auf Grund von Untersuchungen von BANG und ØRSKOV (1937), die ergeben hatten, dass Glukose bei perniziöser Anaemie abnorm schnell in die Blutkörperchen eindringt, und auf Grund der Wirkung von Ventriculin und Leberextrakt wurde angenommen, dass die Wirkung dieser Präparate mit einem Permeabilitätsfaktor in Zusammenhang stehen könne, der möglicherweise mit dem, oder einem der Faktoren identisch ist, die die perniziöse Anaemie zum Verschwinden bringen können.

Bei der weiteren Bearbeitung dieser Frage, sollte zunächst untersucht werden, wie schnell die Wirkung dieser Präparate auf die Permeabilität nachweisbar ist. Hierzu wurden von einer

Reihe normaler Versuchspersonen 3 bis 6 Stunden nach Eingabe des Leberextraktes Blutproben entnommen. In einigen Fällen war eine merkbare Verlangsamung der Permeierung von Glukose schon nach 3 Stunden und eine stärke Wirkung nach 6 Stunden feststellbar. Bei näherer Untersuchung zeigte sich aber, dass man, um sichere Schlüsse ziehen zu können, zunächst die Permeierung einige Tage lang zu entsprechenden Zeiten wie die Blutprobe nach Eingabe des Leberextraktes entnommen worden war, messen muss, da bei einigen Versuchspersonen recht wesentliche Tagesschwankungen in der Eindringungsgeschwindigkeit der Glukose gefunden wurden. Die Permeierung der Glukose war in diesen Fällen früh morgens am schnellsten und am Nachmittag langsam. Unter Berücksichtigung dieser Schwankungen kann man aus den Versuchen schliessen, dass die Wirkung nach 3 Stunden nicht als sicher angesehen werden kann, während die 6 Stunden nach Eingabe des Leberextraktes gemessene Wirkung mit Sicherheit ausserhalb der Fehlergrenze liegt.<sup>1</sup>

Es lag nahe andere Präparate mit antiperniciöser Wirkung zu untersuchen, und wir haben mehrere Leberinjektionspräparate und ausser Ventriculin auch Pylorin und Intricula geprüft. Ausserdem wurden ausführliche Versuche mit Extrakten von Bierhefe vorgenommen, da verschiedene Untersuchungen gezeigt haben, dass Hefe die perniciöse Anaemie beeinflussen kann, was besonders aus einer neueren Arbeit von WINTROBE (1939) deutlich hervorgeht.

In den meisten Fällen wurde nach Eingabe einer einzelnen Dosis des Präparates die Wirkung so lange verfolgt, bis die Anfangswerte der Glukosepermeierung wieder erreicht waren, worauf zum Vergleich ein neues Präparat gegeben wurde. Auf diese Art konnten zahlreiche Versuche mit einer relativ kleinen Anzahl von Versuchspersonen (fast ausschliesslich männliche Medizinstudenten) durchgeführt werden. Es besteht natürlich die Möglichkeit, dass die Reaktion nach einem neuen Präparat noch von dem zuvor gegebenen Präparat beeinflusst wird. Da die Präparate bei den verschiedenen Versuchspersonen in verschiedener Reihenfolge eingegeben werden, und da viele Präparate wiederholt an der gleichen Versuchsperson untersucht worden sind, sind die Versuche trotzdem brauchbar, selbst wenn man auch vielleicht genauere Versuchsergebnisse durch Einfügung

<sup>1</sup> Es sei jedoch darauf hingewiesen dass die Blutprobe, etwa 24 Stunden bei Zimmertemperatur vor der Untersuchung steht.

grösserer Pausen zwischen den einzelnen Versuchen hätte erreichen können.<sup>1</sup>

Ausser Leber- Ventrikel- und Hefepräparaten wurde auch die Wirkung einer Reihe von Vitaminen untersucht. Einerseits sind nämlich die erstgenannten Präparate zweifellos vitaminhaltig, und andererseits zeigte sich, dass die Wirkung der Leber- und Hefepräparate durch kurzzeitiges Kochen stark herabgesetzt wird. Obwohl die angewandten Präparate nur Spuren von C Vitamin enthalten, wurde zunächst die Wirkung dieses Vitamins untersucht und ein sehr kräftiger Einfluss auf die Permeierung der Glukose gefunden. Die Mehrzahl der untersuchten Vitamine hatten eine Wirkung auf die Permeabilität für Glukose, und einige reine Präparate beeinflussten ausserdem die Permeierung von Thioharnstoff.<sup>2</sup>

Ausser Vitaminpräparaten wurde der Einfluss von Fleischextrakt und Eisen untersucht.

Die Resultate der meisten Versuche sind in der folgenden Tabelle zusammengestellt. Da die Wirkung auf die Permeierung am deutlichsten sichtbar ist, wenn die Konzentration von Glukose in den Blutkörperchen noch gering ist, d. h. während des Beginns der Permeierung, ist nur  $K_1$  aufgeführt, welches der Permeierungsgeschwindigkeit entspricht, bis soviel des untersuchten Stoffes in die Blutkörperchen gedrungen ist, dass die Konzentration des Stoffes in der wässrigen Phase des Blutkörperchens 25 % der in der Suspensionsflüssigkeit vorhandenen Menge beträgt. (Bezüglich der Methode sei auf frühere Arbeiten hingewiesen (ØRSKOV 1935, MELDAHL und ØRSKOV 1940 und ØRSKOV 1941 a). Als Mass für den Grad der Wirkung und für die Wirkungsdauer sind für jeden Versuch zwei Zahlenwerte angegeben. Der erste bezeichnet die maximale prozentische Herabsetzung von  $K_1$  für Glukose und der folgende, die Anzahl Tage nach Eingabe des Präparates bis die maximale Herabsetzung von  $K_1$  auf die Hälfte gefallen ist. In einem Teil der Versuche finden sich neben diesen Zahlen für Glukose kursiv gedruckte Zahlen, die die entsprechenden Veränderungen von  $K_1$  für Thioharnstoff angeben. In

<sup>1</sup> Einzelne Versuche wurden wegen spontaner Schwankungen der Permeabilität nicht berücksichtigt. Das Auftreten derartiger Schwankungen ist bei den vielen Stoffen, die die Permeierung beeinflussen, nicht verwunderlich. Es ist vielleicht mehr erstaunlich, wie konstant die Permeierung bei der Mehrzahl der Versuchspersonen ist.

<sup>2</sup> In den meisten Versuchen ist die Permeierung von Glukose, Thioharnstoff, Glycerin, Ammoniumacetat, Hexamethylenetetramin, und Malonamid untersucht.

vielen Versuchen wurde nur eine Bestimmung der Permeabilität für Glukose vorgenommen, da in früheren Versuchen nur für diesen Stoff grosse Veränderungen gefunden worden waren.

Zunächst soll die Wirkung der einzelnen Präparate erörtert, und dann die Wirkung der verschiedenen Stoffe verglichen und diskutiert werden.

Präparat	Dose	Versuchsperson
<i>Leberpräparate per os.</i>		
<i>Extractum fluid. hepatis</i> (A. B.)	10 cm <sup>3</sup>	E. S. 14 (6) J. T. 13 (6) P. B. 13 (4) W. K. 11 (5) P. C. J. 5 (2) H. R. 7 (3) E. AA. 14 (6) W. K. 17 (2) H. R. 8 (>7)
(5 cm <sup>3</sup> = 100 g Leber)	20 cm <sup>3</sup>	E. S. 12 (3) J. T. 15 (4) P. B. 19 (4) W. K. 16 (5) H. R. 22 (3) E. AA. 12 (5)
<i>Campolon</i> (Bayer)	10 cm <sup>3</sup>	E. S. 20 (4) J. T. 10 (>7) P. B. 5(3)
<i>Hepsol</i> (M. C. O.) (10 cm <sup>3</sup> = 50 g Leber entsprechend)	10 cm <sup>3</sup>	P. B. 11 (2)
<i>Exhepa fluid.</i> (Ido) (10 cm <sup>3</sup> = 100 g Leber)	5 cm <sup>3</sup> 10 cm <sup>3</sup> 20 cm <sup>3</sup>	W. K. 7 (5) E. S. 8 (>7) E. Aa. 18 (7) K. G. 12 (3) O E. D. 12 (4)
<i>Exhepa fort.</i> (Ido) (2 cm <sup>3</sup> = 100 g Leber)	4 cm <sup>2</sup>	J. T. 9 (2) G. J. 6 (3) 8 (3)
<i>Exhepa</i> (Ido) (1 Glas (Pulver) = 100 g Leber)	1 Glas	P. L. J. 8 (2) P. C. J. 7 (2)
<i>Leber</i> (in Butter gebraten)	200 g 400 g	J. T. 7 (2) P. C. J. 0 P. B. 5 (7) A. H. 0
<i>Leberpräparate als intramuskuläre Injektion gegeben.</i>		
<i>Campolon</i> (Bayer)	2 cm <sup>3</sup> 4 cm <sup>3</sup>	B. H. 20 (6) C. K. 14 (3)
<i>Exhepa fort.</i> (Ido)	2 cm <sup>3</sup> 4 cm <sup>3</sup>	A. H. 6 (4) E. Aa. 17 (3) E. S. 22 (9) W. K. 8 (6)
auf 100° C erwärmt	2 cm <sup>3</sup>	P. L. J. 4 (3) C. K. 4 (3)
20 Minuten auf 100° C erwärmt	2 cm <sup>3</sup>	A. V. C. 4 (2) O K. G. 3 (3) O E. D. 2 (2) 7(2)
<i>Hepsol fort.</i> (M. C. O.) (1 cm <sup>3</sup> = 100 g Leber)	2 cm <sup>3</sup> 3 cm <sup>3</sup>	A. H. 8 (5) B. A. 10 (5) C. K. 8 (5) W. K. 8 (4)

Präparat	Dose	Versuchsperson
<i>Ventrikelpreparate</i>		
<i>Ventriculin</i> (M. C. O.) (1 Karton 130 g frischen Magen entsprechend)		H. R. 5 (1) E. Aa. 5 (2)
<i>Intricula</i> (Ido) (1 Karton 125 g frischem Magen entsprechend)		J. T. 25 (4) W. K. 15 (4) P. B. 16 (>7)
<i>Bierhefe</i> <sup>1</sup>		
<i>Suspension</i> (150 cc 75 g Hefe ent- sprechend)	150 cm <sup>3</sup>	J. T. 7 (3) P. B. 11 (7) P. C. J. 6 (5) B. H. 11 (>8)
<i>Filtrat</i> 150 cm <sup>3</sup> = 75 g Hefe	150 cm <sup>3</sup>	A. K. 7 (>7) P. L. J. 5 (3) W. K. 8 (6) A. H. 11 (>8) P. L. J. 10 (3) P. L. J. 16 (4)
<i>Filtrat</i> , kurzwierig auf 100° C erwärmt und dann abgekühlt	150 cm <sup>3</sup>	W. K. 3 (2)
<i>Trockenhefe</i> , (autoly- siert) (Ido)	10 g 20 g	J. T. 0 B. H. 0
<i>Vitamin E.</i>		
<i>Ephynal</i> , (Roche) per os Tabl.	80 mg A. V. C. 9 (4) 21 (3) 60 mg K. G. 3 (3) 13 (3) 40 mg H. K. 6 (3) 20 (2) 20 mg E. D. 4 (2) 20 (2) 12 mg A. V. C. 12 (4) 11 (4) 6 mg K. G. 8 (4) 10 (5) 3 mg H. K. 96 (4) 6 (5) 1.5 mg E. D. 3 (2) 10 (5) 1.8 mg K. G. 6 (3) <sup>2</sup> —10 (3) E. D. 8 (4) 10 (2)	
<i>Ephynal</i> , die Tabletten in Wasser gelöst und dann 5 Minuten gekocht	6 mg K. G. 3 (3) 0	
15 Minuten gekocht	6 mg A. V. C. 0 0	
<i>Ephynal</i> , intramuskuläre Injektion	15 mg 30 mg	A. J. 2 (3) 0 E. D. 7 (6) 0
<i>Fertilan</i> (M. C. O.) (Weizenkeimöl)	10 cm <sup>3</sup> Öl 6 cm <sup>3</sup> 3 Tage lang	E. D. 10 (6) 11 (4) A. V. C. 9 (6) —53 (5) G. J. 16 (6) —schwach E. Aa. 16 (4) —schwach

<sup>1</sup> Die Hefe wurde mit Wasser verdünnt, schnell auf 80° erwärmt und dann schnell abgekühlt. Die Hefe wurde teils als Suspension, teils als Filtrat gegeben. 150 cm<sup>3</sup> = 75 g frische Hefe.

<sup>2</sup> — vor der Zahl bedeutet, dass das Präparat schnellere Permeierung bedingt hat.

Präparat	Dose	Versuchsperson
10 cm <sup>3</sup> Öl 24 Stunden mit 100 cm <sup>3</sup> Wasser geschüttelt		E. D. 15 (7) 16 (>7) K. G. 6 (3) 10 (3) A. V. C. 6 (4) 7 (2) A. V. C. <sup>1</sup> 6 (5) 11 (5)
Wässriger Extrakt von 10 cm <sup>3</sup> Weizenkeimöl		E. D. 10 (5) 13 (5) K. G. 8 (5) 19 (5) E. D. 13 (7) 17 (8) E. D. 15 (7) 16 (7) A. V. C. <sup>1</sup> 6 (3) 12 (5)
Wässriger Extrakt von 10 cm <sup>3</sup> Weizenkeimöl auf 100° C erwärmt		E. D. 12 (5) 17 (5)
<i>Lebertran</i>		
Lebertran (standardisiert 500 A und 500 D Einheiten per cc)	25 cm <sup>3</sup>	E. S. 2 (5) E. D. 4 (5)
in 2 Tagen	25 cm <sup>3</sup>	E. Aa. 5 (nicht untersucht) 0
in 3 Tagen	25 cm <sup>3</sup>	G. K. 0 0 J. T. 6 (3) 0
<i>Ascorbinsäure</i>		
Ascorbinsäure (Roche) per os	25 mg	A. H. 0 R. T. 8 (3) 7 (3)
	50 mg	C. K. 8 (3)
	100 mg	K. G. 13 (3) 0 A. V. C. 15 (5) 10 (5) E. S. 8 (4) J. T. 8 (4) A. K. 7 (3)
	200 mg	A. K. 10 (3) K. G. 8 (3) 17 (3) E. D. 27 (6) 7 (3) A. V. C. 10 (4) 8 (2) C. J. 10 (4) 18 (2) H. K. 8 (2) 0 K. G. 6 (3) 13 (2) H. K. 7 (4) 0
	500 mg	A. C. J. 13 (7)
auf 100° erwärmt (in ca 50 cm <sup>3</sup> H <sub>2</sub> O)	100 mg	B. H. 0
	200 mg	W. K. 0
Ascorbinsäure (M. C. O.) in Ampullen à 100 mg per os	100 mg	H. K. 2 (4) 0 E. A. 4 (3) 0
intramuskulär	100 mg	H. K. — 6 (3) 11 (2) E. A. — 6 (3) 0 A. J. — 6 (3) 0 E. D. 9 (3) 7 (2) B. H. 8 (3) 16
<i>Lactoflavin</i>		
Lactoflavin (Ferrosan) per os	1,25 mg	P. J. 3 (2)
	2,5 mg	E. D. 10 (4) 0
	5 mg	G. J. 12 (4) 7 (5)
	10 mg	G. A. 6 (5) 9 (5) H. K. 5 (3) — 7 (4)

<sup>1</sup> Der wässrige Extrakt wurde in diesem Versuch bis zur Trockenheit bei niedriger Temperatur eingedampft. Gehalt an Trochensubstanz weniger als 1 mg.

Präparat	Dose	Versuchsperson
<i>Betaxin</i>		
<i>Betaxin</i> (Bayer)	1,25 mg	S. Aa. N. 3 (2)
(Vitamin B <sub>1</sub> ) per os	2,5 mg	G. J. 3 (6) 0
	5 mg	K. G. 5 (3) 10 (3)
intramuskulär	10 mg	A. V. C. 11 (3) 17 (3) K. G. 14 (4) 6 (2)
	5 mg	G. J. 6 (>5) 8 (4)
	10 mg	A. V. C. 8 (5) 11 (3) K. G. 11 (5) 7 (2)
<i>Becoplex</i>		
<i>Becoplex</i> (Ido) <sup>1</sup> per os	6 cm <sup>3</sup>	P. L. J. 0
<i>Soluchinon</i>		
<i>Soluchinon</i> (Ido) <sup>2</sup>	3 Tabl. 3 Tage lang	A. K. 0 P. L. J. 0
<i>Nicotinamid</i>		
<i>Nicotinamid</i>	25 mg	B. H. 0 0
	50 mg	E. A. 10 (3) 0
	100 mg	G. J. 10 (4) 7 (3) E. A. 4 (4) — 7 (4)
<i>Muskelextrakt</i>		
<i>Muskelextrakt von 200 g schierem Ochsenfleisch</i>		K. G. 12 (>4) 0 E. A. 14 (4) 0
<i>Extrakt auf 100° C erwärmt</i>		T. R. 8 (3) 0
<i>Extrakt von 400 g schierem Ochsenfleisch (+ 1/2 Zitrone)</i>		A. K. 5 (2) E. Aa. 15 (4)
<i>Eisen</i>		
<i>Ferroplex-Tabletten</i> (à 0,25 g Ferrosi tartras)	6 Tabl.	H. K. 4 (>3) 6 (>3)

<sup>1</sup> Becoplex soll sämtliche B-Faktoren enthalten. In 6 cm<sup>3</sup> sollten etwa 9 mg B<sub>1</sub>-Vitamin, 2,1 mg Flavin und 60 mg Nicotinsäure sein.

<sup>2</sup> Jede Tablette enthält 10 mg Na-2methyl, 1,4-naftohydrochinondisulfat.

6 Ferroplex-Tabletten 7 Tage lang (siehe Abbildung 1).

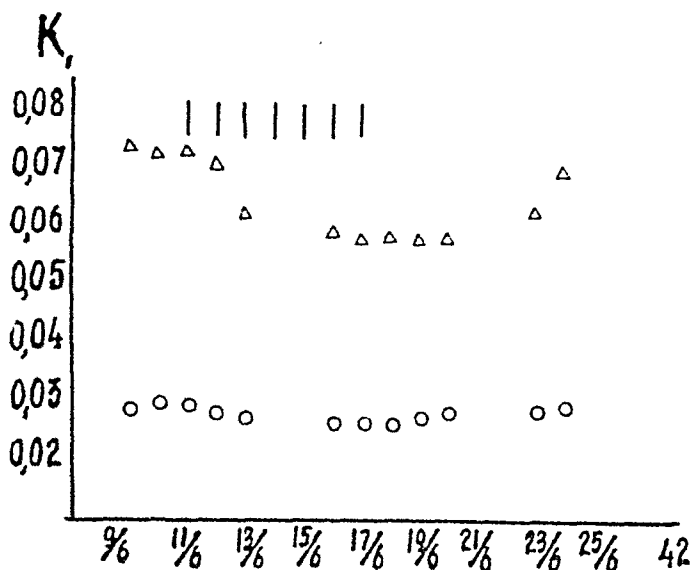


Fig. 1. Die sieben senkrechten Striche bezeichnen die Eisenzufuhr (6 Ferroplextabletten).  $\Delta$  =  $K_1$  für Glukose  $\circ$  =  $K_1$  für Thioharnstoff.

### Leberpräparate.

Die Wirkung der verschiedenen Leberpräparate zeigt grosse individuelle Verschiedenheiten auch für die gleiche Menge desselben Präparates, sowohl was die prozentische Verkürzung von  $K_1$  angeht, als auch für die Wirkungsdauer. Mit allen obenerwähnten Präparaten wurde eine Wirkung nach peroraler Eingabe gefunden, und es lässt sich nicht entscheiden, ob bestimmte Präparate besonders kräftig oder schwach wirken. Auch die injizierbaren Leberpräparate zeigen deutliche Wirkung aber auch hier sind grosse Unterschiede in der Wirkung in den verschiedenen Versuchen vorhanden, und man kann z. B. keine wesentlich stärkere Wirkung nach Injektion von 4 cm<sup>3</sup> als nach 2 cm<sup>3</sup> finden.

Campolon ist mit den übrigen Leberpräparaten schwierig vergleichbar, da nicht bekannt ist, wieviel Leber zur Herstellung des Präparates angewandt wird.

Eine kurzzeitige oder 20 Minuten dauernde Erwärmung des Exhepa fort. auf 100° bedingt eine wesentliche Herabsetzung der Wirkung, die also teilweise auf einem oder mehreren thermolabilen Faktoren beruht. Der antiperniziöse Faktor in der Leber ist an sich äusserst wärmebeständig und verträgt nach BARFRED (1942) 5 Stunden langes Kochen. Die schwache Wirkung, die



nach Einnahme von gebratener Leber beobachtet wird, beruht aber vielleicht zum Teil auf der Zerstörung von wirksamen Faktoren durch die Erwärmung oder auch andere Verhältnisse können hier wohl von Bedeutung sein.

### **Magenpräparate.**

In der Wirkung der beiden untersuchten Präparate wurde ein grosser Unterschied gefunden, und zwar hat *Intricula* eine wesentlich stärkere Wirkung als *Ventriculin*. Die Ursache hierfür liegt wahrscheinlich in den verschiedenen Herstellungsmethoden. Die Wirkung von *Intricula* ist bei Berücksichtigung der Menge des Ausgangsmaterials wesentlich stärker als die verschiedenen Leberpräparate.

### **Bierhefe.**

In den ersten Versuchen wurde die Bierhefe (Weissbierhefe) zunächst mit Essigsäure oder Salzsäure angesäuert und kurzzeitig auf 100° erhitzt. Im Laufe der Versuche zeigte sich jedoch, dass die Wirkung der Hefe und des hiervon gewonnenen Filtrats stärker ist, wenn man bloss auf 80° erwärmt und eine Ansäuerung der Hefe ist nicht erforderlich. Autolysierte Trockenhefe hat keinen Einfluss auf die Permeabilität.

### **Vitamin E.**

Die ersten Versuche wurden mit Fertilan in Kapseln vorgenommen und eine wesentliche Verlangsamung der Permeierung von Glukose gefunden. Auch die Permeabilität für Thioharnstoff war verändert, aber die Eindringung erfolgte hier schneller. Eine neue Packung des Präparates zeigte die gleiche Wirkung für Glukose aber die entgegengesetzte für Thioharnstoff. In den folgenden Versuchen wurde dann immer nur eine Verlangsamung der Permeierungszeit für Thioharnstoff durch Fertilan beobachtet. Das synthetische Vitamin E, Ephynal, bedingt auch eine Verlängerung der Permeierungszeit für beide Stoffe. In der ersten Sendung Kapseln muss also ein Stoff vorhanden gewesen sein, der auf die Permeierung von Thioharnstoff entgegengesetzt wirkt wie Vitamin E. In Fertilan ist ausser Vitamin E ein Stoff vorhanden, der sich mit Wasser ausschütteln lässt und der auch eine Verlangsamung der Permeierung von Glukose und Thioharnstoff bedingt. Dieser Stoff verträgt jedenfalls kurzzeitige Er-

hitzung auf 100° und wirkt in sehr kleinen Mengen. Eine wässrige Lösung mit nur 1 mg Trockensubstanz zeigte schon eine kräftige Wirkung.

Ephynal beeinflusst die Permeabilität schon in Mengen von nur 1.5 mg und eine Steigerung der Dosis von 6 auf 80 mg hat keine Erhöhung der Wirkung zur Folge.

Auch nach intramuskulärer Injektion war eine Wirkung vorhanden.

In einigen Versuchen wurde untersucht, ob Ephynal unter den gleichen Bedingungen, unter denen auch Hefe, Leber und später Vitamin C untersucht wurden (d. h. bei Vorhandensein von Luft) hitzebeständig ist. Die Ephynaltabletten wurden in Wasser suspendiert (etwa 50 cm<sup>3</sup>) und 5 bzw. 15 Minuten gekocht. Nach 5 Minuten war eine schwache Wirkung auf die Permeierung von Glukose und keine Wirkung auf die von Thioharnstoff vorhanden und nach 15 Minuten wurde die Permeabilität für beide Stoffe nicht mehr beeinflusst. Da Ephynal hitzebeständig sein soll, handelt es sich in diesen Versuchen wahrscheinlich um eine Oxydation. Ephynal war in Tabletten an Kieselsäure, Weizenstärke und Talkum adsorbiert und hat deswegen sicher eine grosse Oberfläche.

### Lebertran.

Lebertran hat auf die Permeabilität für Glukose eine schwache Wirkung. Auf die von Thioharnstoff wurde ein Einfluss nicht beobachtet. In einem Falle, in dem 25 cm<sup>3</sup> 3 Tage lang gegeben wurde, war keine Wirkung für Glukose vorhanden.

### Ascorbinsäure.

Ascorbinsäure hat bis zu Mengen von 25 mg eine deutlich messbare Wirkung. Der Einfluss auf die Permeabilität für Glukose ist am stärksten, aber in den meisten Versuchen, in denen die Permeierung von Thioharnstoff untersucht wurde, wurde auch für diesen Stoff eine Wirkung gefunden. In zwei Fällen, in denen die Lösung kurzzeitig auf 100° erwärmt worden war, war keine Wirkung mehr vorhanden, was darauf hindeutet, dass es sich hierbei auch um eine Oxydation während der Erwärmung gehandelt hat.

In fünf Versuchen wurde die Wirkung von intramuskulär injizierter Ascorbinsäure untersucht. In drei Versuchen hatte diese

die entgegengesetzte Wirkung wie die peroral eingegebene Ascorbinsäure.<sup>1</sup> Das angewandte Präparat war von der Firma Medicealco hergestellte Ascorbinsäure in Ampullen, und hatte per os eine nur schwache Wirkung. Diese Versuche haben zweifellos Interesse für die Diskussion der Wirkungsart der wirksamen Präparate.

### **Lactoflavin.**

Schon 1.25 mg Lactoflavin beeinflussen die Permeierung von Glukose kräftig und die von Thioharnstoff weniger sicher. In dem Versuch mit 6 cm<sup>3</sup> Becoplex wurden 2.1 mg Flavin eingegeben und keine Wirkung auf die Permeierung von Glukose gefunden.

### **Betaxin (B<sub>1</sub>).**

In Mengen von nur 1.25 mg hat Betaxin Einfluss auf die Glukosepermeierung (Verlangsamung). Bei Mengen von 5 mg und mehr ist auch Wirkung auf Thioharnstoff (langsamere Permeierung) vorhanden. Intramuskuläre Zufuhr des Präparates hat die gleiche Wirkung. Merkwürdig ist, dass nach 6 cm<sup>3</sup> Becoplex, welche 9 mg B<sub>1</sub> enthalten sollen, keine Wirkung beobachtet wurde. Es wäre von Interesse zu untersuchen, wieweit die Wirkung der Vitamine die gleiche ist, wenn sie in einem bestimmten Mengenverhältnis gegeben werden.

### **Nicotinamid.**

Nicotinamid bedingt langsamere Permeierung von Glukose und Thioharnstoff in Mengen von wenigstens 50 mg.

### **Soluchinon.**

Dieser Stoff, der K-Vitaminwirkung hat, ist ohne Einfluss auf die Permeierungsgeschwindigkeit.

### **Muskelextrakt.**

Der Extrakt ist in folgender Weise hergestellt. Feines schieres Fleisch wird 4 mal durch eine elektrisch getriebene Fleischmaschine gepresst und dann mit Wasser und Alkohol vermischt,

<sup>1</sup> Wiederholung der Injektionsversuche mit einer neuen Packung Ampullen ergab eine langsamere Permeierung von Glukose.

sodass der Alkoholgehalt etwa 60 Prozent wird. Die Mischung steht bis zum nächsten Tage und wird dann filtriert. Der Alkohol und ein Teil des Wassers werden in Vakuum bei Erwärmung auf weniger als 40° abgedampft. Bei Eingabe von einer 200 g Fleisch entsprechenden Menge ist eine erhebliche Verlangsamung der Permeierung von Glukose vorhanden. In zwei Versuchen wurde als Geschmacksstoff der Saft einer halben Zitrone mit eingegeben, aber da in den beiden anderen Versuchen noch kräftigere Wirkung vorhanden war, besteht kein Zweifel, dass die beobachtete Wirkung durch den Fleischextrakt bedingt wird. Kurzzeitige Erhitzung auf 100° verringert die Wirkung nur unwesentlich.

### Eisen.

Eine Untersuchung der Wirkung von Eisen auf die Permeabilität sollte eigentlich schon früher vorgenommen werden. Versuche dieser Art wurden indessen erst in der letzten Zeit begonnen. Eisen bedingt eine deutlich feststellbare Verlangsamung der Permeabilität für Glukose und auch auf die Permeierung von Thioharnstoff ist eine Wirkung vorhanden.

### Besprechung der Ergebnisse.

Die zuvor beschriebenen Versuchsergebnisse haben ergeben, dass nicht allein, wie zuerst angenommen wurde, die antiperniciösen Mittel die Permeierung durch die Membran der roten Blutkörperchen verlangsamen, sondern dass die gleiche Wirkung auch nach Eingabe der meisten der untersuchten Vitamine und Vitaminpräparate, nach Fleischextrakt, wässrigem Extrakt aus Weizenkeimöl und nach Eingabe von Eisen vorhanden ist. Diese Stoffe können nicht mehr unter dem Begriff von antiperniciösen Mitteln zusammengefasst werden, und selbst wenn man die Stoffe unter dem Gesichtspunkt eines Einflusses auf die Neubildung der roten Blutkörperchen betrachten will, können hierunter nicht alle untersuchten Präparate eingeordnet werden.

Man rechnet damit, dass der exogene Faktor der für die Bildung des wirksamen Leberstoffes notwendig ist, in Fleischextrakt, Hefe, Magenpräparaten und Fleisch vorhanden ist. Dieser Faktor soll hitzebeständig sein, was nicht damit in Übereinstimmung steht, dass ein wesentlicher Teil der Wirkung auf die Permeabilität nach kurzzeitigem Kochen der Hefe- oder Leberpräparate

verschwindet. Daher ist es wahrscheinlich nicht der exogene Faktor, der in diesen Extrakten die stärkste Wirkung hat, wenn auch die Hitzebeständigkeit nicht als ein besonders sicheres Kennzeichen aufgefasst werden kann. Die Wirkung von Vitamin E, welches auch als hitzebeständig angesehen wird, ist nach Kochen von 15 Minuten wirkungslos. Vitamin C soll für die Neubildung der Blutkörperchen notwendig sein, aber für die sonst untersuchten Vitamine ist bisher keine sichere Beziehung zur Neubildung der Blutkörperchen nachgewiesen worden. Eisen ist natürlich ein notwendiger Bestandteil der roten Blutkörperchen.

Wenn die Permeabilität und die antiperniciösen Mittel in Beziehung zueinander gesetzt wurden, war die Ursache hierfür, dass BANG und ØRSKOV eine erhöhte Blutkörperchenpermeabilität für Glukose bei perniziöser Anämie gefunden hatten, die nach Behandlung wieder normal wurde, und dass die gleiche Wirkung einer Verlangsamung der Permeierung auch bei Behandlung von normalen Versuchspersonen gefunden wurde (ØRSKOV 1941 (2)).

Man könnte sich ja sehr wohl vorstellen, dass die bei perniziöser Anämie vorhandene schnelle Permeierung nicht durch ein Fehlen des Faktors, der mit der Blutkörperchenneubildung in Zusammenhang steht, bedingt ist, sondern durch einen oder mehrere andere Stoffe. Die Steigerung der Permeabilität könnte auch auf einer Abnormen Zusammensetzung der bekannten Bestandteile des Plasmas beruhen. Bei perniziöser Anämie wird angegeben, dass besonders niedrige C Vitaminwerte im Plasma vorhanden sind (ALT, CHIN und FARMER 1939), und möglicherweise kann man später auch niedrigere Werte von anderen Vitaminen im Plasma nachweisen.

Es ist möglich, dass Faktoren, die für die Neubildung der Blutkörperchen von Bedeutung sind, auch auf die Permeabilität der zirkulierenden Blutmenge Einfluss haben, was aber erst mit Sicherheit nachweisbar ist, wenn man mit den reinen Stoffen arbeiten kann, und wenn gezeigt werden kann, dass diese sowohl Einfluss auf die Blutkörperchenneubildung als auch auf die Permeabilität haben.

Andererseits zeigen die in der vorliegenden Arbeit beschriebenen Versuche, dass Vitamine, von denen eine Beziehung zur Blutkörperchenneubildung nicht bekannt ist, eine deutlich messbare Wirkung auf die Permeierung von Glukose haben.

Besonders zwei Möglichkeiten müssen zum Verständnis der Art der Wirkung aller dieser Stoffe auf die Blutkörperchen in Be-

tracht gezogen werden: 1. eine direkte Einwirkung und 2. eine indirekte Einwirkung.

1. In einer Reihe bisher unveröffentlichter Versuche zeigte sich, dass kleine Konzentrationen von Leberextrakt, Hefeextrakt, Vitamin C, E und B<sub>1</sub> auch in Reagenzglasversuchen eine langsamere Permeierung der Glukose bedingen. Man kann hieraus natürlich nicht mit Sicherheit schliessen, dass die erwähnten Stoffe auch im Organismus die Blutkörperchen beeinflussen, da dort wesentlich andere Verhältnisse vorliegen und u. a. die Konzentration, in der die Stoffe sich im Blute finden, nicht bekannt ist.

2. Es ist von vorneherein sehr wohl möglich, dass die Wirkung auf die Permeabilität der Blutkörperchen eine indirekte ist, da alle die erwähnten Präparate wahrscheinlich physiologisch aktiv sind und daher eine veränderte Konzentration von Hormonen, Vitaminen und anderen Bestandteilen des Plasmas im Blut verursachen können. Es ist merkwürdig, dass eine Reihe von so verschieden wirkenden Stoffen ungefähr die gleiche Wirkung auf die Permeierung der Glukose hat. Dies bedeutet jedoch nicht, dass in allen Fällen die gleiche Änderung der Struktur oder Zusammensetzung der roten Blutkörperchen entsteht, da eine langsamere Permeierung eines Stoffes auf verschiedene Art hervorgerufen werden kann.

Die Injektionsversuche mit Ascorbinsäure, bei denen eine schnelle Permeierung der Glukose auftritt, deuten in hohem Grade auf eine indirekte Wirkung, da man bei peroraler Eingabe eine langsamere Permeierung erhält. Die Wirkung wurde allerdings nur mit einer Packung Ampullen von Ascorbinsäure beobachtet, die per os zugeführt nur eine schwache Wirkung hatten. Es liegt nahe anzunehmen, dass der Unterschied durch eine Beeinflussung des C Vitamins im Darmkanal oder der Leber und damit im Blute bedingt worden ist.

Mit den übrigen Vitaminen erhält man bei peroraler oder intramuskulärer Eingabe gleiche Wirkung.

Die Vitamine wirken in sehr geringen Mengen, die den täglich zugeführten entsprechen, und die Wirkung hält sich mehrere Tage lang.

Die Konzentrationssteigerung der zugeführten wirksamen Stoffe im Plasma ist sicherlich sehr gering. In zwei Versuchen wurde der Gehalt an Ascorbinsäure im Plasma nach Eingabe von 100 mg des Stoffes, der einen deutlichen Einfluss auf die Permeierung von Glukose hatte, untersucht.

	A. K. 11/12 41	E. S. 11/12 41
Anfangswert . . . . .	0.60 mg%	0.60 mg%
Nach einem Tag . . . . .	0.64 mg%	0.60 mg%
Nach zwei Tagen . . . . .	0.65 mg%	0.50 mg%

In einem Versuch ist also eine geringe Steigerung vorhanden, in dem anderen keine Erhöhung und ein späteres Absinken.

Da unser Wissen über die Wirkungsweise der Vitamine im Organismus ja sehr begrenzt ist könnte es von Interesse sein, den Mechanismus des Einflusses dieser Stoffe auf die Permeabilität näher zu untersuchen.

Die Beeinflussung der Permeabilität für Thioharnstoff ist wesentlich unsicherer als die für Glukose. In den meisten Fällen bedingt eine Anwendung des wirksamen Stoffes in grösseren Dosen jedoch auch eine Einwirkung auf die Permeierung von Thioharnstoff.

### Zusammenfassung.

Frühere Untersuchungen haben ergeben, dass Leberextrakt und getrockneter Magen beim normalen Menschen eine langsamere Permeierung von Glukose durch die Membran der roten Blutkörperchen bedingen.

Die vorliegenden Versuche haben ergeben, dass viele andere Präparate die gleiche Wirkung haben und gleichzeitig wurde in vielen Versuchen eine Verlangsamung der Permeierung von Thioharnstoff beobachtet.

Eine Wirkung wurde nach Eingabe folgender Stoffe gefunden: verschiedene Leberpräparate, Magenpräparate, Bierhefe und deren Extrakte, Fleischextrakt und Eisen und nach einer Reihe von Vitaminen: Vitamin C, Vitamin B<sub>1</sub>, Vitamin B<sub>2</sub>, Vitamin E, Weizenkeimöl, sowie ein wässriger Extrakt dieses Präparates, nach Nikotinsäureamid und eine schwache Wirkung auch nach Lebertran.

In Weizenkeimöl kann ein Stoff vorkommen, der eine schnellere Permeierung von Thioharnstoff bedingt.

Die wirksamen Bestandteile in diesen Stoffen wirken wahrscheinlich in kleinen Mengen. Ein Einfluss auf die Permeabilität

wurde nach 25 mg Vitamin C, 1.25 mg Vitamin B<sub>1</sub>, 1.25 mg Vitamin B<sub>2</sub>, 1.5 mg Vitamin E, 50 mg Nikotinsäureamid und nach 1 mg wässrigem Extrakt von Weizenkeimöl beobachtet. In den meisten Fällen wurden geringere Dosen als die hier angeführten nicht untersucht.

Eine Wirkung von Leberextrakt trat 6 Stunden nach der Eingabe auf.

Die wirksamen Bestandteile in der Leber und in Hefe sind teilweise hitzeunbeständig und die Sicherheit dieser Beobachtung wird diskutiert.

Es wird weiterhin erörtert, wie die Beeinflussung der Permeabilität der roten Blutkörperchen vorsieht und sowohl mit einer direkten als einer indirekten Wirkung gerechnet. Als Grundlage hierfür dienen bisher nicht veröffentlichte Beobachtungen über die Beeinflussung der Permeabilität der roten Blutkörperchen nach Zusatz der genannten Stoffe in vitro und einige Versuche mit Vitamin C Injektionen, die schnellere Permeierung von Glukose bedingten, d. h. eine umgekehrte Wirkung hatten wie nach peroraler Eingabe des Stoffes.

Andere Präparate hatten nach intramuskulärer Zufuhr die gleiche Wirkung wie peroral.

Es ist mir eine angenehme Pflicht den Firmen FERROSAN, ALFRED BENZON, MEDICINALCO, F. HOFFMANN-LA ROCHE und BAYER für grosszügige Überlassung von Proben der genannten Präparate meinen besten Dank zu sagen.

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## Studies on the Conducting Properties of the Human Skin to Direct Current.

By

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### Introduction.

GILDEMEISTER's monograph (1928) on the conducting properties of the skin to direct current (d. c.) and alternating current (a. c.), and SCHAEFER's book on electrophysiology (1940) contain comprehensive descriptions of the investigations on this problem. The results are briefly as follows.

The d. c.-resistance decreases after conduction of a constant current for some time through the skin and also with increasing voltage. When the voltage is decreased subsequently, the resistance does not increase to the initial value but to a lower value (hysteresis). The resistance to anodic conduction is different from that to cathodic conduction<sup>1</sup>. In the case of short-lasting current impulses, the skin shows properties similar to a polarization cell or a shunted condenser. The a. c.-resistance of the skin is less than its d. c.-resistance, decreasing with increasing frequency of the a. c. — Finally, the skin gives rise to a phase shift between alternating voltage and -current (voltage after current) in the same way as a polarization cell or a condenser. The subcutaneous and internal tissue of the human organism conducts a galvanic current almost as a low-ohmic resistance.

As the cause of the above mentioned conducting properties, GILDEMEISTER (1928) and his school assume polarization of the skin due

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<sup>1</sup> Anodic or cathodic conduction is defined as a d. c.-conduction through the respective part of the skin with the anode or the cathode, respectively, as different electrode. The indifferent electrode is immersed into an electrolyte bath into which also a large part of the skin, e. g. one arm, is immersed.

to different ion mobility in the cells and the cell membranes with an accumulation of ions on the stratum corneum and, especially, on the cell membranes in the stratum germinativum. The d. c.-resistance of the skin is therefore an apparent resistance, determined by the state of permeability of the cell membranes and varying with their permeability.

MUNK (1873) and GÄRTNER (1882) explained the decrease in d. c.-resistance of the skin depending on the time of conduction by an increase in electrolyte content of the skin due to electro-endosmosis. GILDEMEISTER (1928), however, assumed that this effect must be due to a reversible increase in the membrane permeability of the living cell layers of the stratum germinativum accompanied by a decrease in skin polarization. EBBECKE (1921, 22, and 23) proposed the same explanation for the decrease in d. c.-resistance after mechanical treatment of the skin and after conduction of d. c. through the skin (EBBECKE's mechanical and galvanical reaction); the decrease in d. c.-resistance during the psycho-galvanical reflex should be due to a decrease in polarization on the surface of the sweat glands (GILDEMEISTER 1915). As a basis for these assumptions, GILDEMEISTER pointed out that a simultaneous determination of the d. c.- and the a. c.-resistance of the skin (at frequencies above 840 cycles), before and after conduction of d. c. through the skin, showed a decrease in the d. c.-resistance, only (GALLER 1912, GILDEMEISTER 1915, GILDEMEISTER and KAUFHOLD 1920). Since the a. c.-resistance which GILDEMEISTER regards as the real resistance of the skin, determined by its electrolyte content, remained constant after d. c.-conduction, it must be considered impossible that a change in electrolyte content of the skin occurred in the above described case. MUNK's and GÄRTNER's interpretation is thereby considered disproved.

However, after d. c.-conduction through the skin, EINTHOVEN and BIJTEL (1923) actually proved the existence of a decrease in the a. c.-resistance of the skin at frequencies below 840 cycles, but not at higher frequencies. The same observations were made by ROSENDAL (1940) after moistening of the stratum corneum with an electrolyte. These results may be explained by the fact that the a. c.-resistance of the skin at frequencies above 500—1,000 cycles is almost completely capacitive and is independent of a change in the electrolyte content. The a. c.-resistance at frequencies below 500—1,000 cycles, however, has both a capacitive and an ohmic component which latter decreases with increasing electrolyte content of the skin. It must, further, be emphasized that GILDEMEISTER's high-frequency a. c.-resistance (5,000 cycles) of the skin in series with the internal tissue is predominantly due to the resistance of the internal tissue which is independent of the electrolyte content of the skin, while the low-frequency a. c.-resistance mainly originates from the skin. A change in the electrolyte content of the skin will therefore not be registered by a determination of the a. c.-resistance at frequencies above 500—1,000 cycles (GILDEMEISTER and his school), but exclusively by a determination of the low-frequency a. c.-resistance. Since, however, the last-mentioned re-

sistance shows a decrease after conduction of d. c. and after moistening with electrolyte (EINTHOVEN and BIJTEL, ROSENDAL), MUNK's and GÄRTNER's previously mentioned explanation is further supported and GILDEMEISTER's objection against it is invalidated.

As mentioned above, GILDEMEISTER located the electric resistance of the skin to the stratum corneum and especially to all cell layers in the stratum germinativum, while REIN (1930) thought it localized to the stratum lucidum. LEWIS and ZOTTERMAN (1927) demonstrated that the decrease in d. c.-resistance of the skin in EBBECKE's galvanic reaction must be caused by a lesion of the stratum corneum and that the same decrease could be produced by pricking this layer with a needle. These investigations make a localization of the d. c.-resistance of the skin to the stratum corneum probable. The same localization was found by ROSENDAL (1940) in the case of the a. c.-resistance at frequencies between 30 and 20,000 cycles<sup>1</sup>.

In view of the differing interpretations given in the literature concerning the cause of the decrease in skin resistance after conduction of d. c. and the localization of the d. c.-resistance in the skin, a number of new experiments were performed which might elucidate this question.

### Method.

*The electrodes.* The most suitable electrodes were chosen in agreement with the conditions stated by GILDEMEISTER (1915) and on the basis of some investigations concerning the d. c.-resistance as a function of e. m. f. of the following electrode-electrolyte systems. The d. c.-resistance of the systems was determined at different potentials in a Wheatstone bridge with an error of about 1 per cent.

1) Mercury-calomel electrode.

a) Mercury-calomel electrode with an area of 1.7 cm<sup>2</sup> and a thickness of the calomel layer of 3 mm.

b) Mercury-calomel paste electrode with an area of 1.7 cm<sup>2</sup> and a thickness of the mercury-calomel paste — saturated with KCl-solution — of 1 cm. The paste was prepared by grinding in a mortar 2 cc mercury, 5 g of calomel and 3 cc saturated KCl-solution.

c) Platinum-mercury-calomel electrode with an area of 2 cm<sup>2</sup>. The electrode was prepared as described by BISKUPSKI (1938).

Contact between two of the above described electrodes was obtained by means of a glass tube containing saturated KCl-solution with 2 per cent agar. The resistance of this tube amounted to about 200 ohms.

2) Silver-silver chloride electrode.

a) Platinum-silver-silver chloride electrode with an area of  $\pi$  cm<sup>2</sup> prepared according to BROWN (1934).

<sup>1</sup> As regards the experimental technique and a number of other points concerning the conducting properties of the skin and the internal tissue to a. c., cf. ROSENDAL (1940).

b) Silver-silver chloride electrode consisting of a silver plate  $15 \times 1 \times 0.1$  cm, which was wound as a helix, 1 cm high and 2—2.5 cm in diameter. The silver helix was fastened to a silver wire, 5 cm long, and was then covered with silver chloride by electrolysis of a  $\frac{1}{10}$  N HCl-solution during 10—12 hours at a current of 10 milliamperes. The silver helix was then mounted in a cylindrical ebonite vessel with a basis of 7 cm<sup>2</sup> and a height of 2 cm. The lead went through the bottom of the vessel. Finally, the silver helix was covered by saturated KCl solution with 2—3 per cent agar.

Three separate sets of silver-silver chloride electrodes were investigated. Their resistance was determined for the conduction in both directions (marked in the table: I, II) after immersion of the electrodes into a saturated KCl-solution.

Table 1 shows the resistances of the above mentioned electrode-electrolyte systems measured at various potentials and, furthermore, the potential of the systems themselves.

Table 1.

*Resistance values in ohms to direct voltages between 1.32 and 1940 millivolts.*

Voltage in mV.	Mercury calomel electrode	Mercury calomel paste electrode	Platinum mercury calomel electrode	Platinum silver-AgCl	Silver-AgCl I		Silver-AgCl II		Silver-AgCl III		III 10 days later	
					I	II	I	II	I	II	I	II
1.32		420	190									
6.65	3 840	420	780	70	6.4	5.4			15	11	11	9
13.2	3 840	422	1 370	70	5.9	5.4	4.5	5	14	12	14	8
64.5	3 842	419	2 600	50	5.3				10.5	11		
113	3 850	419	3 300	60	5.3		4.5	5	12	11	9	8
571					5.2	5	4.4		10	10	9	8
1 940					5.3		4.4		10	10	9	8
Voltage in mV of the system itself	0.4		1		0.3		1.5		0.4			

Table 1 shows the high potential-independent resistance of the calomel electrodes. Their high resistance must be ascribed to the calomel layer and therefore these electrodes are less suited for resistance measurements on the skin. The resistance of the platinum-mercury calomel paste electrodes is also potential-independent, but somewhat lower. The resistance of the platinum-mercury-calomel electrodes is to a great extent dependent on the voltage. Hence, this electrode —

in the form described by BISKUPSKI (1938) — cannot be considered unpolarizable and is, therefore, unsuitable for biological measurements. Silver-silver chloride electrodes show a very low resistance in both current directions and they are almost potential-independent. Consequently, these electrodes must be regarded as best suited for resistance determinations on the skin. In the course of the investigations, the electrodes have been tested repeatedly; resistance and voltage of the system itself were found to be constant and of the magnitude given in the table. As the resistance of the object of measurements — viz. the skin — has been above 1,000 ohms in almost all experiments, and since the voltages applied were above 100 millivolts, the influence of the electrodes upon the determination of the resistance of the object was less than 1 per cent.

*The measurements.* Part of the experiments concerning the conducting properties of the skin to direct current were performed as current determinations; the error involved was less than 1 per cent in a circuit consisting of a known source of e. m. f., a galvanometer, and the object of measurements. The object in the present case was a silver-silver chloride electrode in an ebonite cylinder (7 cm<sup>2</sup>) — 1 per cent KCl-solution or saturated KCl-solution as contact electrolyte — the skin on the volar side of a person's forearm which rests upon the electrode — the internal tissue of the same arm, the thorax, and the other arm — and, finally, the skin of the arm which is immersed until the elbow into a vessel containing 1 per cent KCl-solution into which the other silver-silver chloride electrode is also immersed. Since the immersed region of the skin is of a far larger area than the region which is in contact with one of the polarization-free electrodes (7 cm<sup>2</sup>), the resistance of the first mentioned will be very low. The resistance of the object of measurements will therefore almost exclusively be determined by the resistance of the 7 cm<sup>2</sup> of skin, since the resistance of the electrode and of the contact electrolyte can be disregarded. In agreement with this fact, most experiments showed a resistance of the object of measurements higher than 10,000 ohms at 2 volts. After removal of the stratum corneum of the 7 cm<sup>2</sup> of skin — where the skin resistance is located — the same object of measurements showed a resistance of about 600 ohms at 2 volts. The last mentioned resistance, which is exclusively that of the internal tissue and of the immersed area of the skin, is further independent of potential, variations of the direction of current, and of the time of conduction. Therefore, the present method enables us to measure the d. c.-resistance of a known skin area.

In some experiments, the object of measurements was  $2 \times 7$  cm<sup>2</sup> skin area on the volar side of a forearm resting on 2 silver-silver chloride electrodes at a distance of about 5 cm, connected by the internal tissue of the forearm, the resistance of which is 200–300 ohms. The resistance of this object was measured with an error of less than 1 per cent in a Wheatstone bridge.

When studying the time dependence of the current, the d. c. was registered by means of an electrostatic oscillograph (resonance period 1/3,000 sec) and a d. c.-amplifier (BUCHTHAL and NIELSEN 1936).

In another series of experiments, the d. c. was overlaid by a. c. with a frequency of 200 cycles. The a. c.-amplitude was registered as a function of time by means of an oscillograph and a d. c.-amplifier and was compared with the amplitude of a. c. of the same frequency over a known ohmic resistance. Further, an a. c. with a frequency of 1,000 cycles was overlaid by the d. c. through the above mentioned object of measurements. By means of two d. c.-amplifiers, the alternating current and -voltage were then via an electronic switch (Philips) introduced into a cathode beam oscillograph where the standing picture was photographed. The variation with time of the current amplitude was registered and the phase shift relative to the constant amplitude of the alternating voltage was determined.

### Experimental results.

#### 1) *Variations from day to day, individual and regional variations of the skin resistance to direct current.*

In order to elucidate the individual and regional variations of the skin resistance, the d. c.-resistance to anodic conduction at 2 volts was determined on the volar side of the forearm over a period of 6 days on the same region of the skin. It was furthermore determined on 6 different persons and on different regions of the same person. The resistance of 7 cm<sup>2</sup> of the skin at 2 volts and  $\frac{1}{1}$  m KCl-solution as contact electrolyte varies between  $70 \times 10^3$  and  $485 \times 10^3$  ohms on one person and between  $40 \times 10^3$  and  $200 \times 10^3$  ohms on the other.

In different individuals the resistance of 7 cm<sup>2</sup> of the skin at 2 volts and 1 per cent KCl-solution as contact electrolyte varied between  $5 \times 10^3$  and  $250 \times 10^3$  ohms.

The regional variation is shown in table 2.

Table 2.

*Regional variation of the d. c.-resistance in ohms of 7 cm<sup>2</sup> of the skin at 2 volts and 1 per cent KCl-solution as contact electrolyte.*

K. W. ♂ 21 years left arm	Proximal to wrist	Middle of forearm	Near the elbow	Upper arm
Resistance in ohms at 2 volts . . . . .	2 200	82 000	160 000	9 000

The experiments show the great difference in the d. c.-resistance of the skin at 2 volts on the same person from day to day and on different regions, and moreover the great individual

variations. The variations in the d. c.-resistance of the skin must be ascribed to variations in the stratum corneum (cf. section 2) of different persons, of the different regions, and of the same region from day to day.

These variations in the skin-resistance make it necessary that studies on the interdependence of skin resistance and voltage, direction of current, and properties of the contact electrolyte are performed on the same region of the skin and are compared only with resistance values determined in the same experiment.

## 2) *Localization of the d. c.-resistance.*

In recent investigations (ROSENDAL 1940) it was shown that the a. c.-resistance of the skin at frequencies between 30 and 20,000 cycles is exclusively located in the stratum corneum. This was made evident by the decrease in a. c.-resistance of the skin to 0 ohm after abrasion of the skin with emery paper. Histological investigations of the abraded skin region showed further that the lesion was only located to the stratum corneum and not to the stratum germinativum.

In the following experiments, the d. c.-resistance was determined before and after abrading the skin with emery paper in the same way as in the above mentioned a. c.-experiments. The lesion is a maceration of the stratum corneum only, without injuring the corium and without bleedings. The depth of the lesion has not in this case been determined microscopically; as earlier investigations (ROSENDAL 1940) had shown that the abrasion here applied only injured the stratum corneum, it is justified to locate the present lesions to the same layer. Table 3 contains the figures obtained on two persons.

These experiments illustrate the decrease in d. c.-resistance after lesion of the stratum corneum to a value which is of the same order of magnitude as that found recently for the a. c.-resistance of a corresponding object (ROSENDAL 1940).

In agreement with LEWIS and ZOTTERMAN (1927), it is thus shown that the *d. c.-resistance of the skin as well as its a. c.-resistance are located exclusively in the stratum corneum*, while the stratum germinativum conducts an electric current as does the internal tissue. Moreover, the resistance of the internal tissue, either between two skin areas or from one skin area to the other arm or leg immersed into KCl-solution, is independent of the voltage and of the direction of current. As this resistance is also

Table 3.

*D. c.-resistance in ohms of  $2 \times 7$  cm<sup>2</sup> of the skin before and after abrasion of the skin with emery paper.*

(1 per cent KCl-solution as contact electrolyte.)

Test-person	Before abrasion  Resistance in ohms at 2 volts	Direction of current	After abrasion				
			Resistance in ohms at				
			0.154 volts	0.224 volts	1.14 volts	1.5 volts	2 volts
A. S. ♀ 24 years old right forearm	115 500	I	614	608	588	588	580
		II	608	608	588	592	580
T. R. ♂ 34 years old left forearm	15 000	I	257	254	230	229	
		II	220	218	219	218	

independent of the kind and of the concentration of the contact electrolyte, and as it is small compared with the resistance of the skin, it may be disregarded in the following experiments.

3) *Dependence of the d. c.-resistance on the moistening of the skin with electrolyte.*

The studies on the influence of moistening of the stratum corneum either with 1 per cent KCl-solution or with saturated KCl-solution were performed on 10 persons of both sexes. The d. c.-resistance was measured at different potentials between 0.129 and 2 volts. The region investigated was the volar side of the persons' forearm. The resistance was measured during up to 60 min. of anodic and cathodic conduction. In some experiments, the direction of current was reversed after 1 min. or after 2 min.; in other experiments, after 5—10 sec or after 2 min., and the current was then interrupted for 5 min. in between every determination.

All experiments revealed a very marked decrease in d. c.-resistance of the skin, most pronounced after the first 10 minutes of moistening. After the lapse of 30 min., the resistance approximates a constant value which is 5 to 10 times as low as the initial value and which corresponds to a saturation of the stratum corneum with electrolyte. The decrease in resistance is greatest after moistening of the skin with saturated KCl-solution. Some curves of resistance decrease or current increase, respectively, are given in fig. 1.



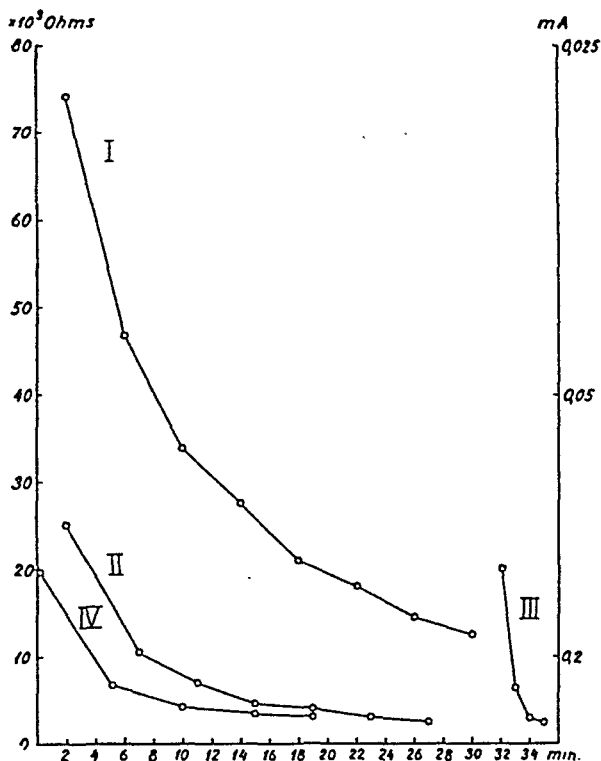


Fig. 1. Dependence of the skin resistance on moistening with electrolyte and on conduction of d. c. at 2 volts.

Ordinates: left, resistance in ohms, right, current in mA. Abscissae: Time in min.

Curve I:  $2 \times 7 \text{ cm}^2$  of skin area, 1 per cent KCl-solution.

Curve II:  $2 \times 7 \text{ cm}^2$  of skin area, saturated KCl-solution.

Curve III:  $2 \times 7 \text{ cm}^2$  of skin area, 1 per cent KCl-solution.

Curve IV:  $7 \text{ cm}^2$  of skin area, 1 per cent KCl-solution.

Curve III was registered after washing of the skin with distilled water, and should be compared with curve II. In curves I, II, and III, the skin resistance to anodic and cathodic conduction for 1 min. was determined, however, only the first mentioned is plotted in the curves. In curve IV, the skin resistance was determined after 5 sec. of conduction and at 5 min. intervals in between every determination.

The decrease in skin resistance is presumably due to an increase in the electrolyte content of the stratum corneum. This is made probable by curve III which represents the increasing resistance obtained after the saturated KCl-solution (experimental curve II) had been washed off from the stratum corneum with distilled water. This explanation is further supported by experiments in which the skin was moistened for 20 min with saturated KCl-solution. In these experiments, an increase in d. c.-resistance of the skin at 2 volts from 10,000 ohms to 18,000 ohms and 19,000 ohms, respectively, in the course of 12–14 min. was found when

the saturated KCl-solution was replaced by 1 per cent KCl-solution as contact electrolyte.

When an e. m. f. above 2—4 volts is applied after the resistance has become constant, a further decrease in the skin d. c.-resistance can be observed. The value then obtained approximates the resistance of the internal tissue. However, this decrease must be due to a short-circuiting of the stratum corneum, as will be discussed in a later section (p. 141).

The experiments described on the foregoing pages indicate that the d. c.-resistance of the skin depends to a high degree on the electrolyte content of the stratum corneum. The investigations of the interdependence between skin resistance and magnitude of the direct voltage, direction of current, and time of conduction therefore involve a considerable error, if the measurements are performed immediately after the electrode is applied. This error can be avoided when the measurements are performed after moistening of the stratum corneum with contact electrolyte for 10 min.

#### 4) *The dependence of the skin resistance on the direct voltage.*

In order to elucidate the voltage-dependence of the skin resistance, the d. c.-resistance in ohms was determined on 7 cm<sup>2</sup> of skin on the forearm at an e. m. f. varying between 0.129 and 2.57 volts and with a 1 per cent KCl-solution as contact electrolyte (fig. 2). The resistance was determined for anodic conduction and, subsequently, for cathodic conduction, each during 1 min. The resistance determination was performed immediately after application of the electrode and was repeated twice at different voltages in the course of 26 min.

In the first experiment (group of curves I), the resistance to anodic and cathodic conduction decreases with increasing e. m. f. from 0.129 to 2.57 volts, if the resistance is measured immediately after application of the electrode. However, the decrease in resistance is no real expression of the voltage-dependence, but must be ascribed to a moistening of the stratum corneum with 1 per cent KCl-solution (cf. p. 137). On repeating the experiment (group II), the resistance is found considerably lower and the voltage dependence disappears in the case of anodic conduction. The reproducible interdependence between voltage and resistance is found after the lapse of 20 min (curves of group III). With increasing voltage from 0.129 to 2.57 volts, the curve indicates

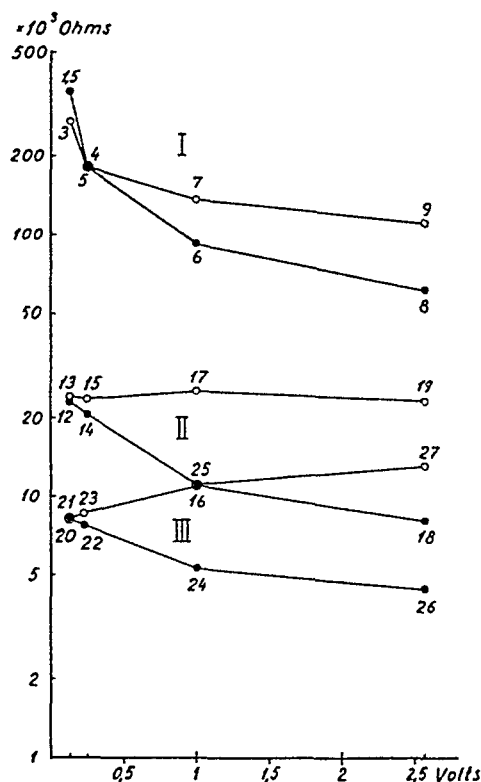


Fig. 2. The dependence of the resistance on the voltage. 7 cm<sup>2</sup> of skin and 1 per cent KCl-solution as contact electrolyte.

Ordinates: resistance in ohms in a logarithmic scale. Abscissae: potential in volts. ○ anodic conduction. ● cathodic conduction. The time of the resistance determination after application of the electrode in min. is given above or beneath the curves.

an increasing resistance to anodic conduction from 8,200 ohms to 13,000 ohms while the resistance to cathodic conduction decreases to 4,000 ohms.

The voltage dependence of the skin resistance at higher voltage is represented in fig. 3. After moistening of the skin with saturated KCl-solution for 20 min., the resistance was determined for anodic and cathodic conduction, during 1 min. each, and at potentials between 0.2 and 12 volts. The voltage dependence between 0.2 and 2 volts was measured in 3 groups of experiments, in the figure marked I, II, III. The voltage dependence up to 12 volts was determined in 2 series of experiments, marked III and IV.

The increase in resistance in the case of anodic conduction and the decrease with cathodic conduction are reproducible as long

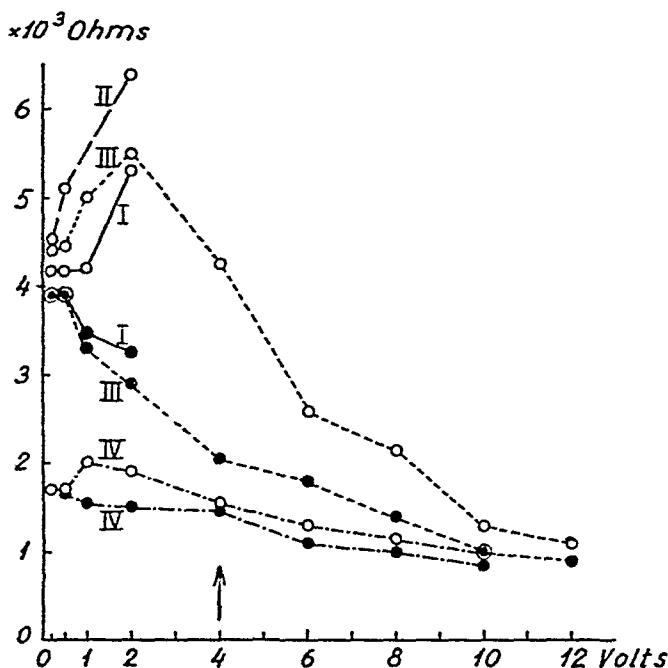


Fig. 3. Voltage dependence of the resistance of 7 cm<sup>2</sup> of skin moistened for 20 min with saturated KCl-solution which serves also as contact electrolyte.

Ordinate: resistance in ohms. Abscissae: potential in volts. ○ anodic conduction. ● cathodic conduction. At the arrow, the person felt pain in the skin region investigated.

as the e. m. f. does not exceed 2 volts (fig. 3, curves I, II, III). At further increase of the voltage (curve III), the resistance both to anodic and to cathodic conduction decreases. On repeating the experiment (curve IV), the increase as well as the decrease in resistance almost ceased, both values now approaching one another and being almost independent of voltage. The decrease in resistance at e. m. f. above 2 volts is accompanied by a marked feeling of pain in the skin, which becomes stronger with increasing e. m. f. Simultaneously, the respective regions become hyperaemic.

The experiments described above reveal a difference in the interdependence of skin resistance and voltage for anodic or cathodic conduction, respectively, at e. m. f. up to 2 volts. Above 2 volts and for both directions of current, the difference disappears, since the skin now behaves like a low ohmic resistance of a magnitude which corresponds to the resistance of the internal tissue. This decrease in resistance is probably due to a short-circuiting of the stratum corneum at voltages above 2 volts. The feeling of pain and the hyperaemia might be correlat-

ed with the formation of histamin in the skin (cf. ROSENTHAL and MINARD's (1939) studies on histamin formation and feeling of pain during electrical stimulation of the skin).

5) *The dependence of the skin resistance on the direction of current.*

As shown in fig. 3, the skin resistance is higher during anodic than during cathodic conduction through the same region of the skin. The difference depends on the voltage and amounts to 350 ohms at 0.2 volts, to 2,600 ohms at 2 volts; at 10 volts, however, the difference is 100 ohms, only.

Fig. 4 exhibits further evidence for this observation. The experiments reveal that *the resistance to anodic conduction at 2 volts is about 60—100 per cent higher than the resistance to cathodic conduction*, irrespective of the initial direction of conduction, and independent of the duration. The difference is greatest in the case of saturated KCl-solution as contact electrolyte. This phenomenon was observed on different regions of the forearm and on 10 different persons.

Fig. 5 shows clearly that this phenomenon just as the other d. c.-conduction phenomena must be located in the stratum corneum. The skin resistance to anodic and cathodic conduction was determined before and after abrasion of the stratum corneum with emery paper, and the difference in resistance was found to vanish as soon as the stratum corneum was injured. The same applies when the stratum corneum is short-circuited by e. m. f. above 2 volts (cf. fig. 3, p. 141).

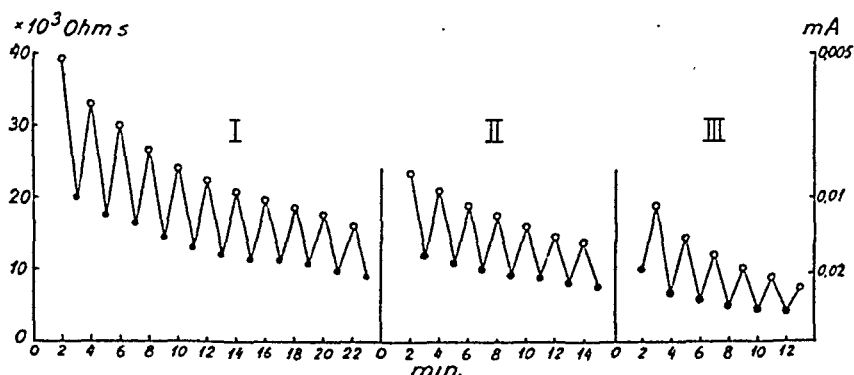


Fig. 4. Dependence of the resistance on the direction of conduction at 2 volts. 7 cm<sup>2</sup> of skin moistened for 6 min with 1 per cent KCl-solution (I) and with saturated KCl-solution (II) and (III). Experiment II begins with anodic conduction, experiment III with cathodic conduction.

Ordinates: left, resistance in ohms; right, current in mA. Abscissae: time in min. ○ anodic conduction. ● cathodic conduction.

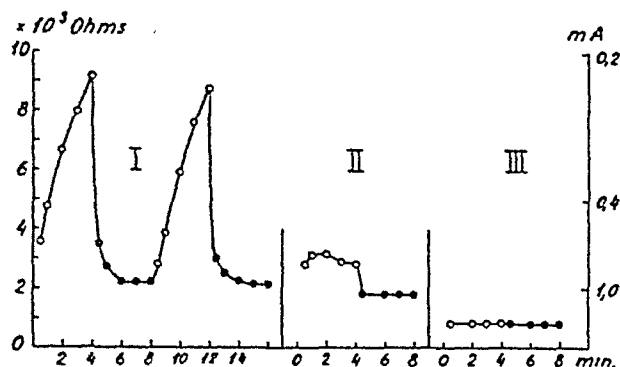


Fig. 5. The resistance of  $7 \text{ cm}^2$  of skin and the internal tissue before (I) and after (II) abrasion with emery paper, and after further abrasion (III).

Ordinates: left, resistance in ohms; right, current in mA. Abscissae: time in min.  $\circ$  anodic conduction.  $\bullet$  cathodic conduction.

The *time dependence* of the skin resistance to anodic and cathodic conduction (2 min in each direction) was investigated partly by registering the current by means of a galvanometer (sensitivity  $10^{-5}$  Amp), partly by registration of the potential difference with an electrostatic oscillograph. Fig. 6 shows a continuous increase in resistance to anodic conduction and a rapid decrease in resistance during cathodic conduction. When the anodic and cathodic conduction are continued more than 2 min, respectively, the resistances approach a constant value.

In some other experiments, the d. c. through the object was overlaid by a. c. with a frequency of 200 or 1,000 cycles. Also at low frequency a. c. the resistance — which has an ohmic resistance component — was found to vary in the same sense as

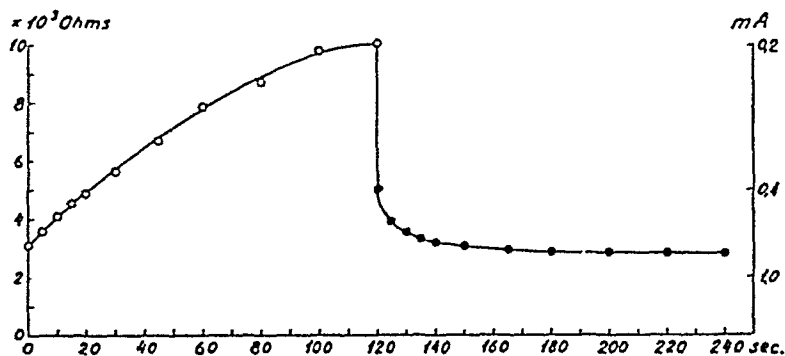


Fig. 6. Time dependence of the resistance at 2 volts of  $7 \text{ cm}^2$  of skin moistened for 20 min with saturated KCl-solution.

Ordinates: left, resistance in ohms; right, current in mA. Abscissae: time in sec.  $\circ$  anodic conduction.  $\bullet$  cathodic conduction.

the d. c.-resistance with the time of anodic as well as cathodic conduction. The a. c.-resistance at 1,000 cycles which is almost exclusively a capacitive resistance did not show any variations.

6) *Investigations of the pH of the contact electrolyte before and after conduction of d. c. through the skin.*

REIN (1926) found that anodic conduction of d. c. through the skin caused an increase in pH of the contact electrolyte. During cathodic conduction, the pH decreases in the case of males and increases in the case of females. REIN has given no details concerning the pH of the electrolyte before the experiments or whether application of the electrolyte on the skin changed its pH.

In order to elucidate these phenomena, the pH of the contact electrolyte was determined before and after application of the electrolyte on the skin and, further, before and after anodic and cathodic conduction of d. c.

The pH was measured by means of a glass electrode and a valve voltmeter; the error of this determination did not exceed 0.03 in the pH value. (Only Jena glass was used).

The pH of 4 cc of a 1 per cent KCl-solution was measured before and after standing on the volar side of a forearm. The liquid with a mean pH of 5.73 was kept in a cylindrical vessel with a contact area of 7 cm<sup>2</sup>. 14 experiments on 4 persons showed a mean decrease in pH of 0.76, to a mean constant value of pH 4.97, when the pH was measured up to 45 min after standing. The change in pH is most considerable during the first 20 min of standing.

The decrease in pH of the contact electrolyte is not due to an escape of CO<sub>2</sub> from the skin into the electrolyte; furthermore, an increase in pH of the contact electrolyte was found after cleaning of the skin with water, soap, and alcohol in two cases. These observations might indicate that the decrease in pH after standing of the electrolyte in contact with the skin is caused by acid substances liberated from the skin. This explanation is in agreement with SCHADE and MARCHIONINI's (1928) investigations, who found the mean pH of the skin surface to be 3.78.

These observations indicate that the variations of the pH after conduction of d. c. through the skin can first be determined after the pH of the contact electrolyte has reached a constant value in the course of 20—30 min.

Table 4 contains the pH values before and after anodic and cathodic conduction of d. c. at voltages up to 10 volts through 7 cm<sup>2</sup> of skin on the volar side of 4 persons' forearms. The pH values given in the table were obtained before and after the pH value of the solution had become constant after standing in contact with the skin for 20—45 min. At the highest voltage, the current did not exceed 1.65 mA. The last column of the table shows the maximum change in pH of every individual experiment after conduction of d. c.

Table 4.

*pH of the electrolyte in contact with the skin before and after conduction of d. c.*

Person	Electro-lyte	pH before experiment	pH after 20—45 min of standing	pH after 5 min of d. c.-conduction						mA	Max-imum change in pH
				anodic		cathodic					
				4 V	8 V	2 V	4 V	6 V	10 V		
T. R. left forearm	sat. KCl										
	6 cc	4.97	5.22	5.27							+ 0.05
	do.	5.09	4.98	4.91							— 0.07
	do.	4.71	4.91	4.89			4.88				— 0.03
	1 % KCl										
	4 cc	5.48	5.03	5.03	5.12						+ 0.09
H. F. right forearm	do.	5.94	4.39	4.37	4.39					1	0
	do.	5.66	4.88				4.88	4.88	4.83	1.65	— 0.05
	do.	5.30	5.49			5.43	5.42	5.40		1.35	— 0.09
	do.	5.89	5.67	5.63			5.63			0.55	— 0.04
	do.	5.71	5.74	5.73			5.74			0.95	— 0.01
	do.	6.67	5.68	5.61			5.64				— 0.07
Mean value											+ 0.05

It can be seen from the table that the highest variation in pH of the contact electrolyte is 0.09, the mean variation being 0.05. During anodic conduction, the pH value increased in 2 experiments, decreased in 5 experiments, and remained unchanged in one experiment. In the case of cathodic conduction, the pH decreased in 5 experiments and remained unchanged in one experiment.

As a result of the pH determinations it was found that electro-



lyte in contact with the skin changes its pH from about 5.73 to 4.97 in the course of 45 min. Conduction of d. c., however, is of no appreciable effect on the pH of the contact electrolyte.

### Discussion.

The experiments described above confirm LEWIS and ZOTTERMAN's (1927) localization of the d. c.-resistance of the skin to the stratum corneum and they are also in agreement with ROSENDAL's (1940) localization of the a. c.-resistance of the skin to the same layer. In contrast to the view held by most of the previous investigators — GILDEMEISTER and his school —, the various conductivity phenomena must be located in the stratum corneum. The assumption can no longer be maintained that the d. c.-resistance of the skin is an apparent resistance due to polarization corresponding to the cell membranes in the living cell layers of the stratum germinativum.

Presumably, the d. c.-conduction through the stratum corneum occurs through the excretory ducts of the sweat glands or the sebaceous glands and along the hair sacks; this was made probable by REIN's investigations (1926) concerning the transport of coloured cations and anions (methylen blue and eosin) through the skin. However, the probability exists that an ion migration also takes place directly through interspaces between the horny cells of the stratum corneum. The cause of the large individual and regional difference in the d. c.-resistance of the skin might be found in variations of the length, dimensions, and electrolyte content of these channels through the stratum corneum.

The decrease in d. c.-resistance of a skin area of 7 cm<sup>2</sup> either after a lesion of the stratum corneum by abrasion with emery paper or by short-circuiting at an e. m. f. above 2—6 volts is due to the formation of electrolyte-filled low ohmic shunt resistances in the stratum corneum which form a contact with the well-conducting internal tissue through the living cell layers of the stratum germinativum. It is natural to explain the decrease in the d. c.-resistance after EBBECKE's mechanical and galvanic reaction in the same way. EBBECKE's galvanic reaction (1921) was accompanied by a dilatation of the vessels, which EBBECKE ascribed to a formation of dilating substances during the passage of d. c. through the skin. On the basis of ROSEN-

THAL and MINARD's (1939) investigations it may be assumed that the dilatation of the vessels and the feeling of pain which appear in the respective skin area at potentials above 2—6 volts are caused by the formation of histamin during the passage of d. c. through the skin.

Moistening and conduction experiments show the significance of the electrolyte content of the stratum corneum for the d. c.-resistance of the skin, in agreement with earlier observations when low-frequency a. c. was applied (cf. p. 131).

The increase in electrolyte content of the stratum corneum in the course of the conduction also explains the hysteresis phenomenon (cf. p. 130) which GILDEMEISTER assumed to be caused by a change in polarization of the living cell layers.

Finally, the increasing electrolyte content of the stratum corneum due to moisture and conduction of d. c. may explain the decrease in d. c.-resistance of the skin during anodic and cathodic conduction which appears with increasing voltage up to 2—4 volts. In the recent literature, even in SCHAEFER's book on electrophysiology (1940), the decrease in skin resistance with increasing voltage is interpreted as a reduced polarization of the skin at higher voltages. However, this interpretation is erroneous, and it would also be a misinterpretation to consider the decrease in resistance at voltages above 2—6 volts, which are due to a short-circuiting of the skin, as an expression of reduced polarization.

The voltage dependence of the skin resistance determined after moistening of the stratum corneum with 1 per cent KCl-solution or saturated KCl-solution shows — in contrast to earlier investigations — that the resistance to anodic conduction increases with increasing potential up to 2—4 volts, while the resistance to cathodic conduction decreases. The difference between the resistances in both directions approaches zero at 0.1 volt, while at 2 volts the difference can exceed 100 per cent of the resistance to cathodic conduction. The difference in resistance to the two directions of current is an expression of a polarity of the stratum corneum to d. c. The polarity is independent of the initial direction of the current and it increases with time during conduction in both directions, since the resistance decreases or increases, respectively, until a constant value is obtained after 2—3 min of conduction in each direction. A polarity was also found in the case of a. c. of a frequency of 200 cycles where the resistance is partly an ohmic, partly a capacitive re-

sistance; it could, however, not be found at 1,000 cycles when the conduction is only capacitive.

REIN (1926) found a similar difference in skin resistance to anodic and cathodic conduction when 1/100 m or 1/10 m KCl-solutions were applied as contact electrolyte. He interpreted the difference as an expression of a change in polarization of the skin after reversion of the current.

According to investigations of the polarity of organic membranes (BETHE and TOROPOFF 1914, 1915, MICHAELIS and coworkers 1925—27, FREUNDLICH 1930, and HÖBER 1936), an electronegatively charged membrane is mainly permeable to monovalent anions. The polarity can be reversed by re-charging the membrane. In the case of an electronegatively charged, water-free collodion membrane, BETHE and TOROPOFF observed an alkali formation on the anode side, and a formation of acid on the cathode side of the membrane after conduction of d. c.

REIN (1927) has put forward the view that the membrane effect of the skin must exclusively be located to the stratum lucidum and that it is due to a negative charge of this layer; the membrane should, further, be more permeable to anions than to cations, and the outward migration of anions should be more difficult than the inward migration. REIN refers to the fact that water permeates the skin cathodically when the anode is placed on the outer side of the epidermis and the cathode on its inner side. However, the opposite direction of conduction has not been studied. REIN points out further that the living cell layers of the stratum germinativum and the hair follicles are better stained by basic methylen blue with a staining cation than by acid eosin with a staining anion. Since, however, the stratum corneum is not stained by any of these substances, the experiments do not give any information concerning the electric conduction of this layer, but they show only that the stratum germinativum is best stained by methylen blue. Finally, REIN mentions the observation previously discussed of an alkali formation in the contact electrolyte beneath the anode after conduction of d. c. through the skin, and this is considered to indicate that the skin is negatively charged; BETHE and TOROPOFF's investigations of the pH variations on each side of an electronegatively charged collodion membrane are taken to support this view. However, the writer's investigations do not confirm REIN's change in pH of the contact electrolyte after conduc-

tion of d. c. through the skin; therefore, and moreover in view of the above discussed objections, it is the writer's opinion that the conditions determining the electric charge of the skin are not yet elucidated.

This view-point is supported in a paper by BRAUNER (1930). In the case of an electronegatively charged, non-living, semi-permeable membrane, BRAUNER found the opposite polarity for d. c. to that in the skin, and he explained this effect by a pH variation in alkaline direction on the anode side and in acid direction on the cathode side. As a membrane, he used the seed-coat of the horse-chestnut. The d. c.-resistance of this membrane at 2.5 volts and during cathodic conduction is 315 per cent of the resistance during anodic conduction, when  $\frac{1}{4}$  N  $K_2SO_4$ -solution is used as an electrolyte on each side of the membrane. Since the polarity of this membrane to d. c. is opposite to the polarity of the skin, we cannot for them both expect the same change in pH on the anode- and cathode side of the membrane as a cause of their polarity. Consequently, it must be assumed either that this explanation is erroneous, or that the skin is not electronegatively charged.

Also other facts seem to indicate that the electric charge of the stratum corneum is more complicated than assumed by REIN. SCHADE and MARCHIONINI's (1928) observation of an acid reaction on the surface of the skin are in favour of an electro-positive charge on the outer side of the stratum corneum. The same authors found an alkaline reaction (pH 7.44) after lesion of the epidermis which might indicate an electronegative charge on the inner side of the stratum corneum.

Apart from the electric charge of the stratum corneum, the ion size of the electrolytes on the outer- and inner side of the stratum corneum will be of importance, since the resistance to anodic conduction is determined by the migrating-in of the anions and -out of the cations, while the resistance to cathodic conduction is determined by the opposite transport. This view has been further supported by preliminary experiments in which solutions with large and small anions, respectively, and different cations were used as contact electrolytes. It was found that 10 per cent sodium benzoate solution and 10 per cent sodium citrate solution as contact electrolyte annulled the difference in skin resistance to anodic and cathodic conduction, while 1 per cent NaCl led to a similar difference as 1 per cent KCl-solution.

Finally, also the concentration of the contact electrolyte is of importance, since the difference in skin resistance at the two directions of current increased from 45 per cent to 200 per cent when saturated KCl-solution was applied as contact electrolyte instead of 1 per cent KCl-solution.

It may, therefore, be assumed that the polarity of the skin to d. c. can be ascribed to the electric charge of the stratum corneum, however, also the type and the concentration of the contact electrolyte are of significance.

The results from a closer study of these phenomena will be given in a later publication.

### Summary.

The resistance to direct current (voltage 0—12 V) of a 7 cm<sup>2</sup> skin area on the volar side of the forearm has been determined under different conditions, using polarization-free silver-silver chloride electrodes.

The skin resistance is almost exclusively located in the stratum corneum and decreases very markedly with increasing electrolyte content of this layer. The conductivity of the stratum germinativum corresponds to that of the internal tissue which is rich in electrolyte, and behaves like a low ohmic resistance to d. c.

On 7 cm<sup>2</sup> of skin area, the stratum corneum shows polarity to d. c. at currents below 1 mA, since the resistance to anodic conduction increases with increasing e. m. f. up to 2—4 volts, while the resistance to cathodic conduction decreases. At voltages above 2—4 volts, the resistance to both directions of current decreases to a value which corresponds to the resistance of the internal tissue. This decrease in resistance is presumably due to a short-circuiting of the stratum corneum. The polarity of the stratum corneum, which is highest when voltages around 2 volts are applied, is brought into relation to a possible electric charge of the stratum corneum and to the type and the concentration of the contact electrolyte.

The pH of the contact electrolyte (4 cc 1 per cent KCl-solution) decreased after standing on the skin for 20—45 min to a mean value of 4.97, determined on 4 persons in 14 experiments. In contradistinction to REIN's investigations, no change in the pH

of the contact electrolyte after anodic and cathodic conduction of d. c. through the skin could be observed.

My heartiest thanks are due to F. BUCHTHAL, M. D., for stimulating advice and helpful discussions in the course of this work.

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## **Muscle Action Potentials during Electric and Chemical Stimulation of the Motor Area of the Cerebral Cortex in the Waking Animal and under Anaesthesia.<sup>1</sup>**

By

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By investigating the potential-differences occurring in voluntary and reflex contraction an attempt has been made to contribute, amongst other things, to the elucidation of the mode of action of voluntary and reflex innervation.

It might perhaps seem more natural to gather information about these matters by leading off the electrical phenomena directly from the central nervous system. But investigations of the action potentials of the individual muscle fibres during contraction in response to cortical stimulation offer a possibility of studying a more localised activity in the motor areas of the cerebral cortex. When using the electro-encephalogram as an indicator of activity in the central nervous system, most investigators have employed diffuse leading off from larger areas, and have thus obtained an interference picture which it is often difficult to correlate with the function of the individual ganglion cell. Of recent years, however, some few investigations have been made with localised leads (microelectrode (FORBES)).

A well-tried method is available for local leading off from individual muscle fibres in situ. And even though the results will be

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more complicated, since the way from the cortex to the muscle fibre involves a number of unknown quantities, certain inferences may still be drawn with respect to the function of the individual ganglion cell.

As is well known, the motor ganglion cell of the anterior horn of the medulla forms a motor unit together with the axis cylinder and a number of muscle fibres ranging from 30 to 300 (SHERRINGTON). The impulse frequency in such a motor unit is determined by the ganglion cell and is as a rule transferred without change of frequency to the muscle fibres, so that it will usually be possible to determine from the action potentials in the individual muscle fibre the impulses issuing from the spinal ganglion cell belonging to it. Exceptions to this rule are found in peripherally released muscle action potentials, e. g. in myotonic muscles and veratrin-poisoned muscles; in these special cases it is not possible to draw conclusions from the muscle action potentials as to the impulse frequency in the motor cells of the spinal ganglia. Investigations by HOEFER and PUTNAM (1939) and BUCHTHAL and CLEMMESSEN (1941) have shown that the individual motor units in normal muscles work independently of each other. These authors examined the simultaneous function in various parts of a muscle, leading off with three different needle electrodes to three different amplifiers and three oscillographs, and only found transient synchronous activity from different fibres in normal muscles, while this was the rule in certain pathological conditions (neurogenous atrophy of spinal origin). In contrast with this, SEYFFARTH (1940) found a certain interdependence between the frequency of different motor units in normal muscles; as simultaneous registration from different parts of the muscle was not made in these experiments, the value of this observation seems rather doubtful.

The release of impulses from motor ganglion cells in the anterior horn may take place partly from the motor area of the cerebral cortex, partly by the release of reflexes. It may then be asked whether conclusions can be drawn from the muscle action potentials as to the function of the motor cortex. From various quarters it has been pointed out (e. g. by HOFFMANN 1928, HOEFER and PUTNAM 1939) that there is no direct connection between the frequency in electric cortical stimulation and the frequency of the muscle action potentials resulting from them, since even the smallest muscular contraction is accompanied by a reflex action



on the spinal ganglion cell, by which the frequency of the muscle action potentials may be changed. Attention should no doubt be paid to this fact; but, as will appear from the sequel, certain conclusions may be drawn from the muscle action potentials as to the function of the central nervous system.

Investigations on muscle contraction or muscle action potentials in response to chemical or electric stimulation of the motor area of the cerebral cortex have been made by a number of authors (inter alia BERTHA 1927, CÜPPERS and MÜSCH 1940, DUSSER DE BARENNE 1929—41, and SUNDBERG-TRENDELENBURG-VOSS 1928). Investigations of the action potentials of a single or some few muscle fibres, as is possible by means of a concentric needle electrode (ADRIAN and BRONK 1929), have, however, as far as we know, not previously been made during electric or chemical stimulation of the cerebral cortex. We have therefore in a series of experiments, partly on the waking animal, partly under superficial and deep anaesthesia, investigated the action potentials from the individual muscle fibres during chemical and electric stimulation of the motor area of the cerebral cortex.

### Experimental Technique.

Only adult rabbits both males and females were used for the experiments (weight 3—3½ kg). The animal is placed on a heated operating table, the surface of which is covered with a copper plate connected to earth. Under quite superficial ether anaesthesia a large unilateral craniotomy is performed and one cerebral hemisphere exposed. The dura is split and turned back, and the brain is covered with compresses saturated with body-warm physiological saline, which is not removed until the beginning of the experiment. — Now the extensors of the opposite forearm are exposed. By leading off from these muscles with a concentric needle electrode it will only be possible to register the action potentials of the single muscle fibre in very feeble contractions; with moderate and stronger contractions there will be some overlapping of potentials from the neighbouring fibres. In order to avoid this overlapping we partially cut through the muscle group proximally to the electrode as well as the motor nerve (cp. ADRIAN and BRONK 1929). The musculature is covered with bodywarm saline compresses. The animal is left till wide awake (determined by means of the cornea reflex measured quantitatively with v. Frey's Reizhaare (irritating hairs) (JUUL 1940). The concentric needle electrode is connected to an amplifier a. m. BUCHTHAL-NIELSEN with a time constant of 1/10 sec. The action potentials are registered partly by means of a cathode ray oscillograph, partly by means of an electrostatic oscillograph. They could, in addition, be led off to a loud speaker so that during the whole

experiment the muscle action potentials could be heard or seen, as desired. In the experiments under anaesthesia the depth of the anaesthesia was determined quantitatively by means of the cornea reflex, with v. FREY's irritating hairs. The frequency of the recorded muscle action potentials as well as the time registration were measured with a measuring microscope.

For electric stimulation of the motor area of the cerebral cortex sinus-shaped oscillations were employed from an impulse generator with frequencies from 6 to 5 000 cycles. At low frequencies, however, the shape of the current will be somewhat distorted by the transformer mentioned below. Two bent platinum electrodes c.  $\frac{1}{2}$  mm distant from each other, applied with slight pressure to the motor area of the cerebral cortex, were used for stimulation. To prevent stimulus escape shielded wires from the impulse generator were used, and a transformer was inserted before the stimulation electrode; finally good contact was made between the animal and the operating table, which was connected to earth, just as an electrode connected to earth was placed in the muscle proximally to the needle electrode. — Having localised the centre for the exposed muscle group in the cerebral cortex by electric stimulation, chemical stimulation of the motor cerebral cortex was performed by applying a small piece of filter-paper (c. 1 sq. mm.) corresponding to this place, saturated with the solution we wished to investigate. It is of importance that upon exposure of the cerebral cortex as little bleeding as possible should occur, since extensive bleeding will greatly compromise these experiments.

### Electric Stimulation of the Motor Area of the Cerebral Cortex.

The experiments were made partly on non-anaesthetised rabbits, partly on rabbits under ether anaesthesia.

#### a) On the Waking Animal.

In a series of experiments we have tried to determine the threshold stimulus for the evocation of action potentials in the corresponding muscle groups by stimulation of the motor area of the cerebral cortex by electric impulses of varying frequency. With the experimental arrangement used, varying results were obtained from animal to animal, but it was still beyond doubt that a stimulation frequency of 70–100 cycles must be conceived as the optimal stimulation frequency for the form of current employed. In similar experiments CÜPPERS and MÜSCH (1940) found an optimum at 70 cycles. The average threshold stimulus for the various frequencies will appear from Fig. 1.

In this connection it may be of interest to recall that in previous experiments (JUUL 1939) we have found that the threshold stimulus for the crossed extensor reflex by electric stimulation of n. ischiadicus with a sinus-shaped alternating current likewise has its optimum at a stimulation frequency of 70—100 cycles.

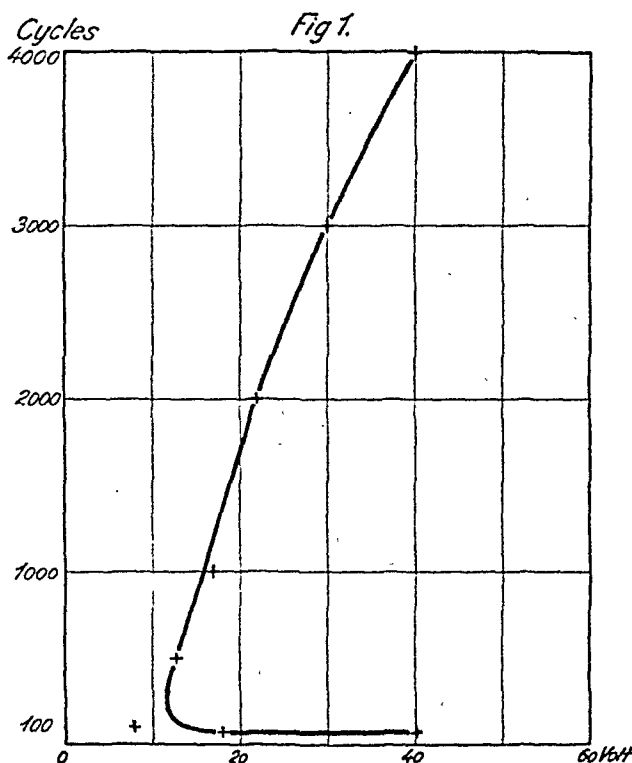


Fig. 1. Threshold stimulus in volt for the evocation of action potentials in corresponding muscle groups by electric stimulation of the cortical motor area with different frequencies.

In connection with these experiments we noted that the muscle action potentials evoked by electric stimulation of the cerebral cortex often persisted for some time, up to several minutes, *after cessation of the stimulus*, even after the electrode had been removed from the cortex. CÜPPERS and MÜSCH (1940) also mention this "after-activity", which they found by direct observation of the muscle contractions. With the experimental arrangement used here this "after-activity" could be registered, while simultaneously with the electric stimulation, in spite of extensive cutting of the motor nerve, we generally got so many different potentials that the frequency could rarely be determined with certainty; the

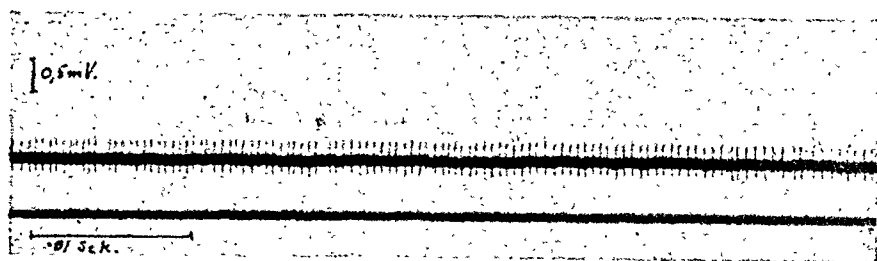


Fig. 2. After-activity after stimulation with 50 cycles. Time 1/100 sec.

frequency and duration of the above-mentioned after-activity seem to be quite independent of the stimulation frequency employed down to about 10 cycles; if a still lower stimulation frequency was used (e. g. 6 cycles), clonus occurred, corresponding to the stimulation frequency employed. If a higher stimulation frequency was used, the after-activity was registered with a frequency of muscle action potentials ranging from a few per sec. to 30—40 per sec.

In order to decide whether this after-activity was of central or of peripheral origin (proprioceptive reflexes), the following experiments were performed. Direct stimulation of the motor nerve or the muscle itself produces no after-activity; this also applies to stimulation of the spinal cord after cutting of the posterior roots, whereas stimulation of the motor cerebral cortex after cutting of the posterior roots produced distinct after-activity. After injection of novocain-adrenalin ( $1\frac{1}{2}$  %) round the concentric needle electrode in the muscle, a distinct after-activity was likewise seen upon stimulation of the cerebral cortex. *Thus the after-activity is not due to proprioceptive reflexes*, but must be of central origin, a conclusion which is also supported by the fact that it mostly disappears under light ether anaesthesia (see below).

#### b) Under Ether Anaesthesia.

Ether anaesthesia strongly affects the muscle action potentials during electric stimulation of the cortical motor area. The most striking feature is the considerable inhibitory effect which the anaesthesia exercises on the action potentials. While, as already mentioned, constant action potentials were observed in the waking condition during the electric stimulation, and as a rule pronounced after-activity after cessation of the stimulus, this



Fig. 3. "Volleys" during stimulation with 20 cycles in light ether anaesthesia.  
Time 1/100 sec.

after-activity often disappeared in quite superficial ether anaesthesia, and at the same time "rhythmisation" was often observed during the stimulation, the action potentials occurring in groups ("clonus"). Under light surgical anaesthesia (cornea reflex absent) we often obtained merely such "volleys" of potentials with completely free intervals (cp. strychnine in ether anaesthesia). Under deep anaesthesia these volleys as a rule diminished in frequency and finally ceased entirely. The frequency of the volleys occurring during anaesthesia was usually about 1—2 per sec., irrespectively of the stimulation frequency employed. To elicit the volleys, increasing intensity of stimulation was required with increasing depth of anaesthesia. Under deep ether anaesthesia it was as a rule impossible to evoke action potentials in spite of strong electric stimulation of the cortex. Fig. 3 is an example of "volleys" under light ether anaesthesia.

## Chemical Stimulation of the Motor Area of the Cerebral Cortex. Strychnine.

### a) In the Waking Animal.

Strychnine nitrate in a 1 % solution was used and a filter paper saturated with strychnine was placed on the motor area of the cerebral cortex. After some minutes' duration partly clonic, partly tonic contractions were observed in the corresponding muscle groups. The contractions as a rule lasted  $\frac{1}{2}$ —1 minute, they occurred repeatedly at about 5 minutes' interval and sometimes lasted for several hours. As a rule it was possible to limit the contractions to a particular muscle group or at least to a single extremity.

The registered muscle action potentials generally show a quite constant frequency, which, however, varies in the different fibres; this impulse frequency often persists for several minutes after the actual contractions have ceased ("central excitatory state" — see below). Shortly before the cessation of the action potentials a decreasing frequency is observed for a few seconds, after which the action potentials in accordance with the all-or-none principle disappear abruptly, their magnitude not decreasing but keeping absolutely uniform right up to the abrupt cessation (see Fig. 4).

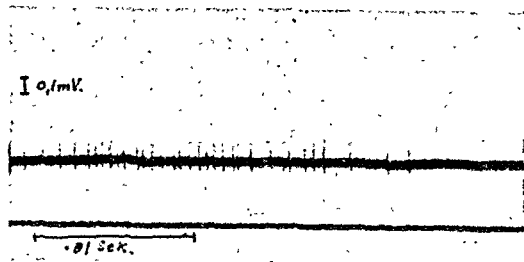


Fig. 4. Last potentials in the "after-effect".

Sometimes pronounced rhythmisation was observed, the action potentials occurring in groups ("clonus"), as a rule at intervals of 1—2 seconds; in certain cases there was complete rest during the intervals; in other cases a constant frequency of the action potentials with smaller deflections between "clonus" was observed. These smaller action potentials are probably due to neighbouring fibres. A "rhythmisation" such as that mentioned may also be seen in the electroencephalogram during strychnine action on the cerebral cortex (DUSSEY DE BARENNE 1941). FISCHER and LÖVENBACH (1934), when leading off from the area striata during strychnine action, also found pronounced rhythmisation. In our experiments the magnitude of the action potentials varied from c. 0.1 to 0.5 mV.

In these experiments the frequency of the action potentials as a rule ranged around 15—50 per sec., but varied from c. 5 per sec. right up to over 100 per sec. In a single experiment we have even for barely 1 second measured an average frequency of 114 per sec., and on a closer analysis of this case it was found that within 1/6 second the frequency rose from 72 to 100 to 118 and finally right up to 125 per sec. then falling again to 118—105—87 and 63. Such a rise in frequency with an ensuing fall ("volleys") was often observed; but only in this one case have we registered this considerable frequency (125 per sec.), which is considerably above the maximal frequencies of muscle action potentials previously observed as initiated by central impulses. An investigation

of the innervation frequency in normal muscles in voluntary contraction is only possible in feeble contractions and there lead to a frequency of 5—20 per sec. (ADRIAN and BRONK (1929), SMITH (1934), LINDSLEY (1935), JALAVISTO and others (1938). In atrophic muscles, on the other hand, where the encroachment of neighbouring fibres is reduced, it is possible to register the frequency in maximal contraction, and the frequencies found there in voluntary contraction were 45—70 per sec. (SEYFFARTH (1938), BUCHTHAL and CLEMMESSEN (1941)).

Next we have investigated whether the strychnine contractions mentioned above were affected by calcium or quinine, substances which in recent years have been employed with success in various affections of the muscles, e. g. Myotonia (HARVEY (1939), BUCHTHAL and CLEMMESSEN (1941)). Calcium was administered intravenously as calcium gluconate (10 %), locally in the muscle around the electrode or imbibed in filter paper applied to the cerebral cortex like strychnine. Quinine was administered in the shape of a 3 % solution of quinine-hydrochloride in a similar manner. No sure effect of calcium gluconate was observed on the action potentials elicited from the muscles by strychnine, whereas 1/2 c. c. quininehydrochloride solution injected around the needle electrode caused a rapid decrease in the frequency of the muscle action potentials; after a few minutes the action potentials entirely or partially ceased, while peripheral stimulation of the muscle still elicited action potentials. The application of quinine to the cerebral cortex was without effect on the muscle action potentials. This agrees with previous investigations e. g. by HARVEY (1939) who found that quinine has several different effects on the striated muscles. After a single stimulus it causes increased contraction with a rise in the action potentials, while in tetanic contraction it reduces or does away with the transmission at the motor end plate, as will appear also from our experiments, since it prevents the transmission of the respective stimuli.

#### b) Under Ether Anaesthesia.

In a number of the above-mentioned strychnine experiments we investigated the influence of ether anaesthesia on the contractions. As a rule the visible contractions ceased already in light ether anaesthesia (corresponding to light surgical anaesthesia) (cornea reflex absent (C—67)). As regards the frequency of the

muscle action potentials registered in superficial anaesthesia, it is quite independent of the anaesthesia; but very often in superficial anaesthesia we saw rhythmisation of the action potentials in the shape of the above-mentioned clonus, the action potentials occurring in groups as "volleys" with rising and falling frequency and entirely free intervals of 1—2 seconds or more. Like the visible contractions the action potentials as a rule also disappeared in light surgical anaesthesia; by pinching the opposite extremity brief clonus could, however, sometimes be elicited again, reflexly. Under deep surgical anaesthesia we have only exceptionally observed muscle action potentials.

### Picrotoxin and Cardiazol.

As in the strychnine experiments, we investigated the muscle action potentials after the application of picrotoxin ( $\frac{1}{2}$  %) and cardiazol (10 %) to the motor area of the cerebral cortex.

Picrotoxin has practically the same effect as strychnine, though the effect of picrotoxin was slightly more feeble; thus the visible contractions were not very pronounced. FISCHER and LÖVENBACH (1934), leading off from the area striata during the action of light on the eye, found that picrotoxin gives a very considerable increase in the excitability of the cerebral cortex.

With cardiazol visible contractions were not obtained, though action potentials could be registered which often showed rhythmisation (clonus). The effect of cardiazol applied directly to the cerebral cortex is thus rather weak with the concentration employed. According to SCHOEN (1926), WINNIWARTER (1937), and BERTHA (1927), this substance principally acts on the sub-cortex and the medulla; like ourselves, however, FISCHER and LÖVENBACH (1934) also observed some effect on the cerebral cortex, for by leading off the action currents here they found them increasing in strength after cardiazol.

Thus it would seem that the effect of strychnine on the cortex is essentially stronger than that of picrotoxin, while this again by far exceeds cardiazol in effect.

### Discussion.

Our experiments with electric stimulation and in some degree also with chemical stimulation of the motor area of the cerebral



cortex have shown, amongst other things, a very distinct after-activity which, in electric stimulation, could be demonstrated several minutes after cessation of the stimulus. This afteractivity is, as shown by the experiments, of central origin. As is well known, the central nervous system is credited with the ability to discharge impulses of an excitatory as well as an inhibitory nature ("central excitatory and inhibitory state"); both inhibitory and excitatory nerves run to the smooth muscles, whereas no inhibitory nerves have been demonstrated to the striated muscles (FULTON (1938)). Excitatory as well as inhibitory impulses have been supposed to be released by chemical stimulation (SHERRINGTON, FULTON (1926) and others). Such chemical stimulation have, however, never been demonstrated, and it is more reasonable to suppose that the function of the central nervous system, similarly to that of the peripheral nerves, is associated with polarisation phenomena round the cell membrane. These phenomena may persist for several seconds or more after a single stimulus (FULTON (1938)) and such a theory is compatible with the long-lasting after-activity observed in our experiments. This after-activity can as a rule easily be brought to an end by anaesthesia, which was indeed to be expected in view of the extreme sensitivity of the cerebrum to anaesthetics.

The frequency of the muscle action potentials does not seem to be dependent on the electric stimulation frequency employed, when the latter is 10 cycles or more. With the use of a lower stimulation frequency clonus of similar frequency was, however, found.

As will appear from the experiments, strychnine produces a violent state of excitability in the motor area of the cerebral cortex. As shown by ADRIAN and BRONK (1929) in reflex experiments, the action potentials occur with a higher frequency in the individual muscle fibre the stronger the stimulation of the medulla is. Presumably it may be inferred from this that a very strong stimulation of the motor area of the cortex, as for instance with strychnine, must give high frequencies of the muscle action potentials. We have in fact often found a high frequency of the muscle action potentials in the strychnine experiments; as we mentioned above, as much as 125 per sec. We were never able to register such a considerable frequency in electric stimulation of the motor cortex. With certain reservations it would thus seem warranted to draw conclusions from the muscle action potentials

to the activity of the cortex, even though a possible transformation of the impulse frequency issuing from the cerebrum in the anterior horn cells must be taken into account.

The effect demonstrated from quinine (injected intramuscularly round the electrode) on the strychnine contractions agrees with the inhibitory influence on repetitive stimulation previously demonstrated as characteristic of quinine.

The effect of cardiazol on the motor cortex was rather slight; it is well known that cardiazol, on subcutaneous injection, acts as an effective toxin to contractions (HILDEBRANDT and others (1937)); but as already mentioned, the point of attack for cardiazol is principally subcortical and medullary, while in accord with our experiments the central effect on the motor cortex is rather slight.

### Summary.

1) We have performed local leading off by means of ADRIAN and БРОНН's concentric needle electrode from the extensors of the forearm of rabbits during direct electric and chemical stimulation of the motor area of the cortex partly in the waking animal and partly under anaesthesia.

2) By electrical stimulation of the cortical motor area agreement between the frequency of the muscle action potential and the frequency of the stimulation is only seen when the latter is below 10 cycles.

3) In electrical as well as in chemical stimulation of the motor area of the cortex a pronounced after-activity was found, often persisting for several minutes. This after-activity is of central origin.

4) The frequency of the muscle action potentials varies during electric and chemical stimulation of the cortex between 2—3 per second and c. 80—90 per second. During strychnine action a frequency of up to 125 per second was found.

5) Quinine has an inhibitory effect on the muscle action potentials (the motor end-plate) produced by the action of strychnine on the cortex. Calcium has no effect on the strychnine contractions.

6) Picrotoxin acts much the same as strychnine on the motor area of the cortex, while cardiazol in a 10 % solution has only a very slight central effect.

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## On the Amount of Reticulocyte Ripening Substances in the Plasma of Various Adult Mammals.

By

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It appears that the rate of ripening of reticulocytes in vitro depends to a great extent on the medium in which the reticulocytes are suspended during the ripening experiments (PLUM, 1942). The reticulocytes can only ripen slowly when suspended in 0.9 % NaCl, but if they are suspended in plasma or in saline to which an organic extract has been added, the rate of ripening is increased and this increase, measured by the monomolecular constant for the reactions, is within certain limits in direct proportion to the concentration of these unknown substances that accelerate the ripening of reticulocytes.

In this paper experiments will be given to determine the quantity of ripening substances in the plasma of various animals in relation to the number of reticulocytes normally present in the blood of the same animals. For the experiments I used rabbit blood cells from animals with pronounced reticulocytosis obtained by causing anemia through repeated blood-letting. As regards the technique and the experimental foundations on which this work is based I refer the reader to previous papers (PLUM, 1942, JACOBSEN and PLUM, 1942).

As it was found that the rate of ripening varied with the rabbits from whom the reticulocytes were obtained, a standard (previously described, PLUM, 1942) was introduced in order to compare similar results, and all the rates of ripening found were related to that standard. A 1 % dilution of a commercial preparation

of liver extract (Hepsol fortior, "MCO") was taken as the standard but in order that the unit should not be too arbitrary I chose the amount of ripening substances in ox plasma as the unit, as the plasma of this animal has the largest quantity of ripening substances among those so far investigated. The ripening index 1.00 indicates that the plasma or solution investigated can ripen reticulocytes at the same rate as ox plasma tested against the same blood corpuscles at the same temperature.

On these assumptions I now will give an account of the variations found in several species of mammals and of the individual variations. The techniques employed in these investigations has already been described in detail (PLUM, 1942).

The plasma to be examined was, unless otherwise stated, obtained by centrifuging freshly taken blood the clotting of which had been prevented by sodium citrate (3.8 %) in the proportion 1:4. This dilution was employed in all cases and even in the ox plasma used as a unit whereas I did not correct for the dilution at the final calculations of ripening index. The samples of plasma were examined as soon as possible or else placed in an ice box. Oddly enough, in spite of the rabbit blood cells being suspended in plasma from another species, I have never observed the phenomenon of agglutination.

The other hematological tests were carried out on freshly taken blood unless otherwise stated.

Some times it was not possible to test the blood samples the same day and therefore I examined the influence of storing on the reticulocyte ripening substances in blood and the influence of clotting too.

## I.

### The Influence of Storing and Clotting on the Reticulocyte Ripening Principle in Blood.

As will be seen from the table 1 I have examined I) plasma, II) serum and III) plasma from samples in which the blood corpuscles had been present during storage. The said investigations were made with blood from humans<sup>1</sup> and rabbits.

In all these experiments I worked with blood that had been prevented from clotting by the addition of 3.8 % sodium citrate in the proportion of 1:4; all the determinations were carried out with blood corpuscles from the same rabbit (No. 148).

<sup>1</sup> Investigations regarding the quantity of ripening substances in plasma from normal people are published elsewhere. (C. M. PLUM, 1942, 2.)

Determinations of plasma stored at various temperatures showed uniformly that whereas plasma stored at 37° had, for 24 hours, lost about 15 % of its ripening ability, plasma at 4° took 48 hours to lose a similar quantity of ripening substances. Therefore if plasma is to be kept for later investigation it ought to be stored in an ice box.

Whereas the changes that occurred in pure plasma during incubation were not significant, large changes are to be seen in the plasma when whole blood is stored. When blood is stored at 4° it is seen that the quantity of reticulocyte ripening substances is reduced by about 40 % after 24 hours. If the blood is stored at 37° the changes are even more significant since I find that the concentration of ripening substances after 24 hours is reduced by about 60 %.

It is worth noting that blood from a rabbit that had been anemized and as the result had a high reticulocyte figure on incubation, suffered a considerably quicker decrease in the quantity of its ripening substances than normal blood. This might indicate that here the reticulocytes used more of the ripening substances than under normal conditions. These circumstances will however be taken up for further investigation.

The figures for serum are 33 % lower than those for plasma. The serum figures depend on the incubation temperature so that the loss at 37° during 24 hours equals the loss of ripening substances in serum incubated at 4° for 48 hours just as in the case of plasma.

Table 1.

*The Influence of Storing Temperature on Changes in the Ripening Index in Plasma or Serum.*

	Stored at temperature	S t o r e d i n		
		0 hours	24 hours	48 hours
<i>Human (♂) (Ret. 8 ‰)</i>				
Plasma . . . . .	4°	0.77	0.70	0.66
Plasma . . . . .	37°			
Whole blood . . . . .	4°	0.77	0.46	0.29
Whole blood . . . . .	37°			
Serum . . . . .	4°	0.54	0.44	0.32
<i>Human (♀) (Ret. 4 ‰)</i>				
Plasma . . . . .	4°	0.76	0.70	0.68
Plasma . . . . .	37°	0.76	0.65	0.62
Whole blood . . . . .	4°	0.76	0.41	0.32
Whole blood . . . . .	37°	0.76	0.27	0.13
Serum . . . . .	4°	0.50	0.42	0.34
Serum . . . . .	37°	0.50	0.32	0.23

	Stored at temperature	S t o r e d i n		
		0 hours	24 hours	48 hours
<i>Rabbit normal (Ret. 25 %<sub>100</sub>)</i>				
Plasma . . . . .	4°	0.65	0.59	0.55
Plasma . . . . .	37°	0.65	0.56	0.52
Whole blood . . . . .	4°	0.65	0.40	0.32
Whole blood . . . . .	37°	0.65	0.25	0.12
Serum . . . . .	4°	0.42	0.35	0.30
<i>Rabbit anemized (Ret. 172 %<sub>100</sub>)</i>				
Plasma . . . . .	4°	0.98	0.90	0.85
Plasma . . . . .	37°	0.93	0.84	0.76
Whole blood . . . . .	4°	0.98	0.40	0.26
Whole blood . . . . .	37°	0.98	0.17	0.08
Serum . . . . .	4°	0.67	0.58	0.48
Serum . . . . .	37°	0.67	0.47	0.31

The practical consequence of the results thus obtained is that the plasma must be used as soon as possible after the blood samples have been taken. If this is not possible, the blood ought to be centrifuged, the plasma sucked off and stored at a low temperature until the next day and *not* longer.

## II.

### The Quantity of Ripening Substances in the Plasma of Several Species of Animals.

I only investigated adult animals that objectively appeared healthy and had a blood picture corresponding to the normal as given in the literature on the subject, Table 2.

As far as rodents are concerned they have been carefully examined and numerous normal figures for them are to be found in the literature; in JAFFE (1931), KLIENEGER (1927) and DONALDSON (1924) among others.

Investigations of the blood pictures of domestic animals are by no means numerous; in the present work I have taken the values given by WIRTH (1931) as the basis for judging the animals' suitability for our experiments, for he has collected the majority of the normal values hitherto mentioned for domestic animals.

Altogether I examined 10 mice, 10 rats, 11 guinea pigs, 15 rabbits, 2 sheep, 7 goats, 9 dogs, 6 cats, 8 horses and 11 oxen.

### Methods.

The investigations made are: 1) The blood picture, 2) Determinations of the ripening index.

The way of taking blood samples was altered according to the size of the animals.

In mice, rats and guinea pigs blood was taken from the inferior vena cava under ether. The clotting was prevented by addition of sodium citrate (3.8 %) in the proportion 1 : 4.

Blood was taken from cats by heart puncture under chloroform. The common hematological test on rabbits were made on blood from the medial ear vein. The ripening index was determined on blood taken by heart puncture without narcosis.

From all other animals the blood was taken from the jugular vein on living animals or soon after killing.

The hemoglobin percentage was determined with Zeiss Ikon Hemometer. The erythrocyte and leucocyte counts were made as usually. When the tests were made on diluted blood, I have corrected for the dilution. The reticulocyte counts and the determination of the ripening index were made as formerly described (PLUM, 1942).

The results of the examinations of the blood picture (table 1 and 2) show no significant deviations from counts found in the literature.

The results of the investigation of the ripening index are to be seen in table 3. The ripening index is here put in relation to the reticulocyte number in promille of the erythrocyte count. Plate 1 shows the ripening index in relation both to reticulocytes in promille and in absolute count.

### Discussion.

The variations in the quantity of ripening substances in plasma for each species of animals show that a low reticulocyte percentage goes with a high ripening index and therefore with a high concentration of ripening substances. The same is seen when the ripening index is put in relation to the absolute number of reticulocytes calculated from the percentage of reticulocytes and the number of red blood cells.

It is found that the relation between reticulocyte number and the ripening index is best expressed the straight line equation:  $x = ay + b$ , where  $y$  is the ripening index and  $x$  the number of reticulocytes,  $a$  and  $b$  being constants. The single determination deviate of course from the line expressed by the equation and I have tried to find whether the absolute numbers or the percentage values gives the smallest deviations. By the method of least



Table 2.  
*Hematological Tests.*

	Hemoglobin		Erythrocyte count	
	Own examinations (Zeiss Ikon)	Normal values in the literature	Own examinations	Normal values in the literature
Mice (10 animals) . . .	82—98 %	KLIENENBERGER: 74—105 %	8.83—9.76 mill.	KLIENENBERGER: 7.4—12.4 mill.
Rats (10 animals) . . .	82—94 %	KLIENENBERGER: 63—120 %	7.23—10.12 »	KLIENENBERGER: 5.9—9.9 »
Guinea pigs (11 ann.) .	79—92 %	JAFFE: 83—110 %	4.65—5.83 »	JAFFE: 4.8—6.4 »
Rabbits (15 animals) . .	79—95 %	JAFFE: 70—90 %	4.64—5.81 »	JAFFE: 5.0—6.0 »
Sheep (2 animals) <sup>1</sup> . . .	69—71 %	SOHANTZ (after WIRTH): 57—93 %	9.04—9.84 »	WIRTH: 8.0—13.0 »
Goats (7 animals) <sup>1</sup> . . .	56—72 %	RANITOVIC (after WIRTH): 58.5 % SCHAURMANN after WIRTH): 84—115 %		
Dogs (9 animals) <sup>2</sup> . . .	65—85 %	WIRTH: 60—80 %	10.24—15.31 »	WIRTH: 10.0—20.0 »
Cats (6 animals) . . .	78—91 %	WIRTH: 50—80 %	6.93—9.04 »	WIRTH: 5.5—8.0 »
Pigs (8 animals) <sup>2</sup> . . .	61—75 %	WIRTH: 55—90 %	9.71—10.37 »	WIRTH: 6.5—10.0 »
Horses (8 animals) <sup>1</sup> . .	67—82 %	WIRTH: 60—80 %	5.12—6.63 »	WIRTH: 5.0—8.0 »
Oxen (11 animals) <sup>2</sup> . . .	65—82 %	WIRTH: 60—80 %	7.08—9.67 »	WIRTH: 7.0—10.0 »
			6.18—8.02 »	WIRTH: 5.0—8.5 »

	Reticulocyte count		Leucocyte count	
	Own examinations	Normal values in the literature	Own examinations	Normal values in the literature
Mice (10 animals) . . .	32—40 ‰	ISAAC: 32—80 ‰	7,640—11,200	KLIENENBERGER: 2,000—31,600
Rats (10 animals) . . .	5—10 ‰	KLIENENBERGER: 30—50 ‰ ORTEN, SMITH: 11—50 ‰	8,840—14,840	KLIENENBERGER: 10,800—18,800
Guinea pigs (11 ann.) .	38—53 ‰	JAFFE: 5—50 ‰	8,640—11,680	JAFFE: 5,600—16,000
Rabbits (15 animals) . .	19—32 ‰	JAFFE: 15—40 ‰	7,620—12,040	JAFFE: 4,500—19,300
Sheep (2 animals) <sup>1</sup> . . .	7—9 ‰	WIRTH: single reticulocytes	9,680—11,240	WIRTH: 8,000—10,000
Goats (7 animals) <sup>1</sup> . . .	4—8 ‰	WIRTH: single reticulocytes	8,840—13,400	WIRTH: 8,000—16,000
Dogs (9 animals) <sup>2</sup> . . .	1—4 ‰	KAHANAWA (after WIRTH): 2—3 ‰	7,880—11,260	WIRTH: 9,000—10,000
Cats (6 animals) . . . .	1—5 ‰	WIRTH: single reticulocytes	9,640—10,480	WIRTH: 10,000—15,000
Pigs (8 animals) <sup>3</sup> . . . .	5—8 ‰	WIRTH: 3—4 ‰	9,040—18,440	WIRTH: 10,000—20,000
Horses (8 animals) <sup>1</sup> . . .	1—5 ‰	WIRTH: single reticulocytes	7,280—10,240	WIRTH: 7,000—10,000
Oxen (11 animals) <sup>2</sup> . . .	0 ‰	WIRTH: 0 ‰	6,040—13,240	DU TOIT (after WIRTH): 5,000—10,000

<sup>1</sup> Dr. N. M. PLUM and Mr. OTTOSEN, veterinary surgeon, of the state Veterinary Serum Laboratory kindly helped me to obtain these blood samples and I tender them my best thanks.

<sup>2</sup> Mr. E. BLOM, veterinary surgeon, The Royal Veterinary and Agricultural College (Institute for Normal Anatomy) kindly helped me to obtain blood samples for which I tender him my best thanks.

<sup>3</sup> Mr. J. MARHESSEN, slaughterer at the Public Slaughter House in the Meat Market, Copenhagen, kindly assisted me in obtaining these blood samples and I tender him my best thanks.

Table 3.

*The Relation between Reticulocyte Number and the Ripening Index in Various Mammals.*

Reticulo- cyte in ‰	Ripening Index	Erythro- cyte in mill.	Reticulo- cyte in ‰	Ripening Index	Erythro- cyte in mill.	Reticulo- cyte in ‰	Ripening Index	Erythro- cyte in mill.
<i>Mice</i>			<i>Rats</i>					
31	0.60	8.92	5	0.82	9.97	40	0.52	4.65
32	0.60	9.26	5	0.86	9.14	43	0.48	5.38
34	0.58	9.12	6	0.87	8.91	44	0.50	4.69
36	0.58	8.99	6	0.86	8.49	45	0.48	5.12
36	0.57	9.76	7	0.83	9.26	46	0.48	4.90
37	0.55	8.83	7	0.81	10.12	46	0.47	4.82
37	0.54	9.34	8	0.81	7.23	48	0.44	4.79
38	0.57	9.31	8	0.80	8.24	52	0.45	5.16
39	0.54	9.14	9	0.78	8.92	52	0.43	5.83
40	0.53	9.43	10	0.76	9.66	<i>Cats</i>		
<i>Rabbits</i>			<i>Sheep</i>			1	0.96	9.98
19	0.77	5.23	7	0.75	9.84	2	0.93	10.27
20	0.77	5.03	9	0.72	9.04	2	0.91	10.37
21	0.78	5.81	<i>Goats</i>			2	0.91	10.12
23	0.74	4.93	4	0.83	13.67	3	0.88	9.86
23	0.73	5.12	4	0.85	11.64	5	0.86	9.71
24	0.72	5.12	5	0.80	15.21	<i>Dogs</i>		
25	0.69	4.87	6	0.78	11.30	1	0.94	9.04
26	0.71	5.22	7	0.76	10.24	2	0.92	7.67
26	0.70	4.73	7	0.75	14.42	2	0.91	7.12
27	0.68	5.36	8	0.72	13.16	2	0.90	7.23
28	0.70	4.64	<i>Oxen</i>			2	0.89	8.46
28	0.69	4.96	0	0.98	6.72	3	0.86	6.93
29	0.66	4.74	0	0.98	8.02	3	0.85	7.66
30	0.66	5.46	0	0.99	7.89	3	0.83	8.16
32	0.63	4.83	0	0.99	7.79	4	0.81	7.89
<i>Pigs</i>			0	1.00	7.14	<i>Horses</i>		
4	0.87	6.21	0	1.00	6.98	1	0.94	7.98
5	0.85	6.14	0	1.02	7.72	2	0.92	8.61
6	0.82	6.43	0	1.02	6.18	2	0.91	7.58
6	0.81	6.06	0	1.02	8.33	2	0.91	7.58
7	0.77	5.95	0	1.02	6.89	2	0.90	8.14
7	0.77	5.12	0	1.04	7.12	2	0.89	9.67
8	0.76	5.98	<i>Guinea pigs</i>			3	0.88	7.08
8	0.74	6.63	38	0.52	4.98	3	0.86	8.24
			39	0.54	5.07	5	0.83	7.87

squares I have found that the standard deviation of  $x$  calculated from  $y$  and the probable values of  $a$  and  $b$  not show significant differences whether  $x$  is calculated absolute or relative figures.

The relation between the ripening index and the number of reticulocytes is not merely observed in the single species but if all the species investigated are taken as a whole a low ripening

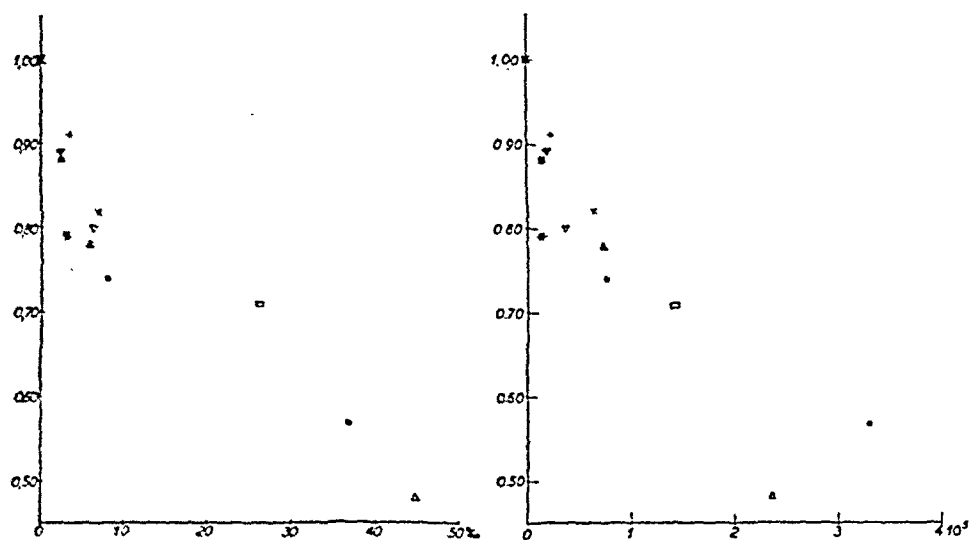


Plate 1.

Survey of the relation between the ripening index and the number of reticulocytes in a number of normal mammals.

Ordinate: Average ripening index.

Abscissa: Average no. of reticulocytes per thousand red blood cells.

Average no. of reticulocytes per. cmm. blood calculated from the number of red blood cells.

- |           |          |                |            |
|-----------|----------|----------------|------------|
| ○ Mice,   | × Rats,  | △ Guinea pigs, | □ Rabbits, |
| ● Sheep,  | ▲ Goats, | ■ Dogs,        | + Cats,    |
| ▼ Horses, | * Oxen,  | * Men.         |            |

index is everywhere found combined with a high reticulocyte figure and vice versa. The same is seen when the ripening index is put in relation to the absolute number of reticulocytes calculated from the percentage of reticulocytes and the number of red blood cells, plate 1.

It is generally assumed that an increased percentage of reticulocytes is due to an increased production of red blood corpuscles. As a fact many experiments, specially clinical and pathological, show that an increased erythropoiesis is accompanied by an increased number of reticulocytes. The present investigation, however, showing the relation between reticulocyte number and ripening index indicates that the amount of ripening substances in the plasma must play a rôle for the number of reticulocytes just as the velocity of the erythropoiesis does. The close relation between the number of reticulocytes and the ripening index seems to indicate that the variations in the quantity of ripening substances must be taken as expressing a biological function and not as merely due to chance.

The highest concentration of ripening substances have been reached in animals that have no reticulocytes as in the case of oxen. Therefore the quantity of ripening substances in this animal's plasma was chosen as the unit for the ripening index. The average of eleven determinations that I made with oxen was fixed as the unit. On comparing this with our standard I found that this (Hepsol fortior, "MCO", of a certain batch in 1 % solution) had a ripening index: 0.97.

### Summary.

The amount of ripening substances in plasma is considerably reduced when whole blood is stored. The reduction seems to be larger, the more reticulocytes were found in the blood sample. When the blood clots about  $\frac{1}{3}$  of the ripening substances disappear.

When plasma is stored only a slight decrease in the quantity of ripening substances occurs. Plasma incubated for 24 hours at 4° remains practically unchanged.

The quantity of ripening substances in the plasma of various mammals shows a certain correlation between the number of reticulocytes in the circulating blood and the concentration of the substances that determine the ripening of the reticulocytes, so that a high percentage of reticulocytes goes with a low ripening index and vice versa. This applies both from individual to individual and from species to species. In blood in which no reticulocytes are found there is the highest concentration of ripening substances. This being the case with oxen I chose to their plasma as the unit for the concentration of ripening substances.

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## **Reticulocyte Ripening Substances in Plasma in Animals with Increased Erythropoiesis.**

By

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In the foregoing paper it has been shown that at fully grown up animals a high reticulocyte number corresponds to a low reticulocyte ripening index and vice versa.

As it shall be shown in the present paper this is not the case under conditions where an argumented erythropoiesis is to be expected, such as in very young animals and in animals after continued blood lettings. In the first case I have used young rats as experimental animals, in the second I have used rabbits.

### **I.**

#### **Ripening Index in the Plasma of Young Rats.**

Investigations of the blood picture during the early days in the life of both human beings and animals are numerous. It is thus generally known that at birth we find a high reticulocyte figure (SEYFARTH and JÜRGENS, 1928; DRABKIN and FITZ-HUGH, 1934; ORTEN and SMITH, 1934; KATO, 1932; and FAXÉN, 1937.)

Young rats were chosen for the investigations because these animals are late in becoming hematologically mature (ADDISON and RICHTER, 1923; CRIMM and SHORT, 1934; DRABKIN and FITZ-HUGH, 1934; JACOBSEN and PLUM, 1942; PLUM, 1942.)

It is difficult to take samples of blood from very young rats and so the following method was chosen for obtaining the material to be investigated, in order to get as much blood as possible: The outer abdominal tissue was cut open and the sample of blood was then taken from vena cava inferior with a graduated syringe fitted with a morphia needle. The amount of blood thus obtainable depended greatly on the age of the animal and so we only put 0.1 c. c. sodium citrate (3.8 %) in the syringe before taking the sample, the remaining quantity of citrate being added afterwards so that the final proportion between the citrate and the blood was 1 : 4. The blood sample was then centrifuged, the plasma sucked off and added to the rabbit reticulocytes in accordance with the method previously described (PLUM, 1942)

In the investigations regarding the ripening of reticulocytes in vitro previously reported (PLUM, 1942), 2 c. c. plasma was used for the experiments in order to be able to carry them out in 4 experimental periods of 0, 2, 4, & 6 hours as described, but it is impossible to obtain blood from young rats in sufficient quantity to be able to carry out the experiments in the usual way and so the technique was altered as follows:

The 8 animals ranged from 0—6 days old and enough blood was obtained from each for 2 samples, one for immediate determination and the other after incubation. The experiments were therefore arranged so that for all the samples the count of the 0 values was made at once and then the loss of reticulocytes in 2 samples was determined after 2 hours, the loss in 3 samples after 4 hours, and in the 3 remaining samples after 6 hours; next the average ripening index figure for the 8 animals investigated was calculated from the average of the results from the various investigation incubation times. The determinations on 6 animals ranging from 8—10 days old were made in the same way, but here it was possible to get enough blood for 3 samples from each, and so the determinations were made as follows: 2 samples after 0—2 and 4 hours, 2 after 0—2 and 6 hours, and finally 2 after 0—4 and 6 hours, and from these the average ripening index figure was calculated. After 10 days enough blood was obtainable from each animal for all 4 samples as in this case there was sufficient plasma (altogether about 1 c. c.) to distribute in all four test tubes. As a matter of course a smaller number of rabbit blood corpuscles was put in each vessel according to the quantity of plasma.

The number of reticulocytes in the freshly taken blood was determined before centrifugation because several investigations, among them those of ORTEN and SMITH (1934), seem to show that the percentage of reticulocytes very closely follows the development of the rest of the general blood picture, and because, in the present work, the figure for the number of reticulocytes was needed for comparison with the quantity of reticulocyte ripening substances in plasma. As appears from Fig. 1., in all

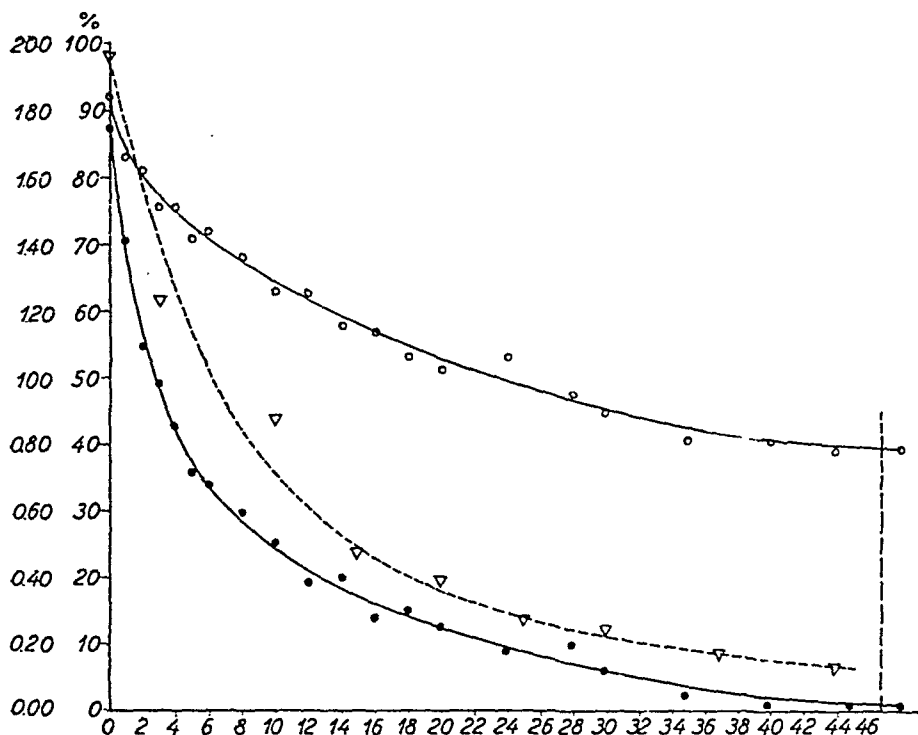


Fig. 1.

Ordinate: No. of reticulocytes in percentage of erythrocytes.  
 \* : Ripening index.  
 Abscissa: Age in days.  
 ○—○: Ripening index.  
 ●—●: Own investigations of the number of reticulocytes.  
 ▽—▽: Orten & Smith's figures for the number reticulocytes.

the young rats investigated, the number of reticulocytes corresponded to that normal for their age.

The results of the determination of the ripening index figures are also given in Fig. 1. As will be seen quite a considerable quantity of ripening substances was found in the plasma of these hematologically not mature animals.

The fall in the quantity of ripening substances takes place parallel with the fall in the number of reticulocytes in percentage of erythrocytes; this fall continues during early life until the young rats get a reticulocyte picture such as is found in mature rats; this happens about 30 days after birth, that is to say at the time when the young rats' blood picture entirely conforms in every respect to that of mature rats.



## II.

**Ripening Index in Animals with Chronic Anemia Induced by Blood-Letting.**

The experiments were made on rabbits. The anemizing can be done by means of an apparatus described by SJÖWALL (1936), but in the present experiments it was done by puncturing the heart daily or every other day and continued if necessary for several weeks.

When it is a matter of a single heart puncture it is easiest to make it with the animal anesthetised, but when it was a matter of daily punctures as in this case, the animal died of pneumonia in the course of a few days. As the results of this experience we changed to operating without an anesthetic and this presented no difficulties.

With the numerous heart punctures we made we never observed formation of any abscess connected with the wound in spite of the fact that we did not shave away any of the hair or even do any special washing round the spot.

On inducing anemia a rapid rise is observed in the number of reticulocytes until a plateau is reached round which the figures fluctuate. The percentage of hemoglobin and the number of erythrocytes fall and form a plateau (see the figure). After a period of long treatment a premortal fall in the number of erythrocytes and the percentage of hemoglobin is often seen accompanied by great fluctuation in the number of reticulocytes.

In the course of from one to two months the animal will die with all the symptoms of aplastic anemia; in some cases we have had experimental animals that lived longer, but these all got severe edema so that we had to kill them. Post mortem examinations showed, presumably as result of hypoprotein-anemia, large subcutaneous accumulations of fluid, as much as 1 litre of fluid in the abdominal cavity and about 200 ccm. fluid in the thorax where the lungs were found to be more or less atelectatic. In addition there was a well-pronounced degeneration of the connective tissue in the liver which is for the matter observed in practically all animals when they have been experimented on for more than 8 days.

Some animals who had been subjected to heart puncture for over a month, besides being in the above mentioned condition showed the wall of the ventricle surrounding the pylorus and the adjoining part of the corpus ventriculi as thin as paper and it broke spontaneously at the dissection.



ced lipemia. Furthermore such rabbits often show symptoms of early stages of edema. General hematological examinations of such rabbits gave pictures that did not differ from those of the other anemized rabbits, but dissection of these animals plainly showed degeneration of the connective tissue of both the liver and the spleen.

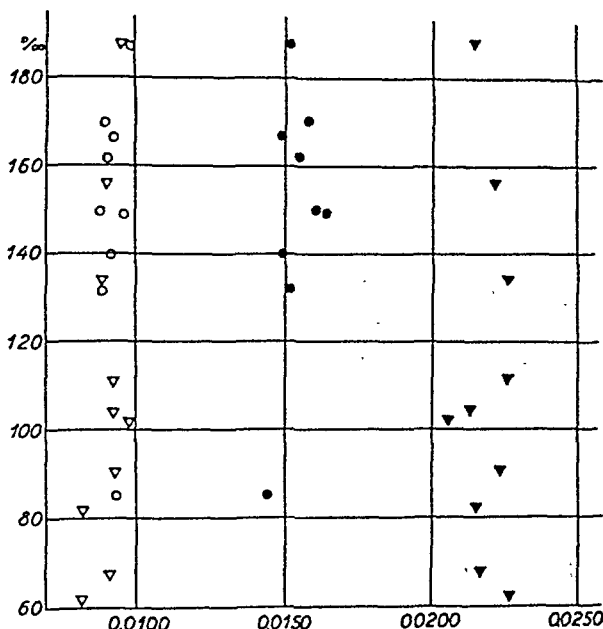


Fig. 3. Variations in the rate of the spontaneous reticulocyte ripening and in the increased rate of the reticulocyte ripening as the result of the addition of ripening substances when the number of reticulocytes varies as the result of anemia induced by blood-letting.

Ordinate: No. of reticulocytes per thousand.

Abscissa: The rate of ripening expressed by the equation for the rate of the reaction of the monomolecular constant.

△ Rabbit 145 } Ripening constant for the spontaneous ripening.

○ " 148 }

▲ Rabbit 145 } Ripening constant for the increased ripening as the result of

● " 148 } added ripening substances (Hepsol fortior 1 %).

These changes occur most quickly if too much blood is taken, especially at the beginning of the induction of the anemia. Therefore too much blood must not be taken during the first days, but the quantity slowly increased; thus 15 c. c. should be taken to begin with and then the quantity should be slowly increased to 50 c. c.

If the heart punctures are stopped a further rise in the number of reticulocytes is often observed the next day, after which a

fall occurs accompanied by a rise in both the hemoglobin percentage and in the number of erythrocytes, and in the course of 2—3 weeks the animal is practically restored, SCHWARZ, 1940.

During the process of inducing anemia no changes appear in the rate of the ripening of the reticulocytes, when exposed to the same amount of ripening substance. Fig. 3 depicts the result of investigations regarding the ripening of reticulocytes obtained from two different anemized rabbits; experiments were made at intervals with blood corpuscles from these animals, suspended in saline and standard solution. From the results it will be seen that the rate of the ripening of the reticulocytes does not vary with the number of the reticulocytes, the degree of anemia, or the length of time during which anemia is induced.

As regards the ripening index, it will be seen from Fig. 2 that this rises. The ripening index rises to a plateau that appears simultaneously with the rise in the number of reticulocytes and falls to normal when the blood-letting ceases in the same time as is necessary for the animal to be restored. This is therefore contrary to what is observed in normal animals where a high reticulocyte figure goes with a low ripening index and vice versa.

### Discussion.

From these experiments reticulocytes appear not to change their character, since they ripen at the same rate when subjected to the same concentration of ripening substances irrespective of the degrees of anemia and of reticulocytosis.

It also appears that the concentration of ripening substances in plasma rises when anemia is induced. From this it may be concluded that reticulocytes in the vascular system of anemized rabbits must be developed about 20—30 % more rapidly than those in normal rabbits. It seems evident that the rise in the concentration of ripening substances can be regarded as a compensatory measure on the part of the organism for the loss of blood, like the compensatory hypertrophy of the marrow during chronic hemorrhagic anemia.

### Summary.

The quantity of ripening substances in young rats is large at birth and afterwards decreases with the fall in the number of

reticulocytes in percentage of erythrocytes, and when figure sare reached that accord with those found in mature rats, the rest of the blood picture also resembles that in mature rats.

During chronic hemorrhagic anemia a rise in the number of reticulocytes may be seen in rabbits. The reticulocytes do not change in character as regards the time they take to ripen when they are subjected to the same concentration of ripening substances, but on the other hand an increase in the quantity of ripening substances in plasma is found in anemized rabbits. This increase shows that under the conditions here described the reticulocytes in the vascular system must ripen about 20—30 % more rapidly than those in normal rabbits.

In the two cases investigated an increased erythropoiesis then is followed by an increased amount of reticulocytes ripening substances in the plasma.

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## A Note on the Preparation of Renin and the Appearance of a Heat-Stable Pressor Substance in Renin Solutions.

By

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Since the discovery of renin by TIGERSTEDT and BERGMAN (1898), several methods for its preparation have been described (HESSEL, 1938, PAGE and HELMER 1939, COLLINGS et. al. 1940, SCHALES, 1942, and others). In order to reduce the number of stages in the preparation, we have adopted certain modifications of our previous method (EULER and SJÖSTRAND, 1941), leading to a crude product of good stability, suitable for the preparation of hypertensin (angiotonin), and serving as a material for further purification. Some observations with regard to the biological actions of renin solutions obtained in this way will also be shortly reported here.

### Preparation of Renin.

Fresh pig kidney cortex was finely ground and extracted for about 1 hour with 2 volumes of cold acetone and filtered on a suction funnel. The residue was allowed to dry in air and was powdered in a mill. 1 kg of the dry powder was extracted with 5 l of 2 % ammonium sulphate in the cold for 2 hours and the solution decanted and pressed off through cloth. The remaining cake was again extracted with 3 l 2 % ammonium sulphate and the solution filtered and pressed off. To the combined filtrates (A) glacial acetic acid was added to 2 % and the precipitate filtered off in the cold after  $\frac{1}{2}$  hour. The clear filtrate (B) (pH about 4) was then evaporated in vacuo until the volume was brought down to  $\frac{1}{20}$  of the original volume. At this point more than 90 % of the renin activity was precipitated, the filtrate

(C) being almost inactive. After filtering through paper, the solids were transferred to a cellophane bag and dialyzed against running water for 24 hours. After dialysis, the contents of the tubes were centrifuged, leaving a potent, slightly coloured, clear solution. After the addition of a small quantity of tricresol, this crude solution has shown no loss in activity even after several months in the ice chest.

If the filtrate (B) was concentrated to  $\frac{1}{10}$  of the original volume, about  $\frac{1}{2}$  of the renin activity was carried down and the rest could be recovered from the filtrate (C). Not even concentration to  $\frac{1}{16}$  of the primary volume was enough, since about 25 % of the activity was found in the filtrate (C). It appears from this and other experiments that the salting out by means of ammonium sulphate requires not only a certain high concentration of the salt but also sufficiently small volume, i. e. a high concentration of renin. On the other hand, activity may be carried down at lower degree of saturation in concentrated solutions. For a satisfactory yield with this method of preparation it is essential that the reaction at the time of precipitation should be about pH 4. In an approximately neutral solution the yield was reduced to about  $\frac{1}{2}$ , and in more acid solutions the yield was also smaller. The activity of the solutions was tested on the atropinized cat's blood pressure under chloralose anaesthesia and also by the formation of hypertensin (angiotonin) with globulines from horse's serum, using the same method as described by EDMAN, EULER, JORPES and SJÖSTRAND (1942). Generally an amount of the renin solution corresponding to 0.1 g dry powder pr kg gave a clean rise in blood pressure of some 20—50 mm Hg. We have observed rather wide variations in the biological effect, however, with preparations of known activity in regard to the ability to form hypertensin. Thus it seems worthy of notice that, in a group of cats at the beginning of autumn 1942, hardly any effect was obtained on the blood pressure, even with doses of 0.4 g dry powder pr kg. Purified dry preparations, usually giving a good response in 0.5—1 mg per kg, were likewise almost ineffective in doses of up to 5 mg per kg. In these animals hypertensin (angiotonin) obtained from horse's serum produced its usual biological action. Whether the lack of effect is due to a deficiency in hypertensinogen in these animals or to other causes, we do not know. In later experiments a good response was obtained with the same extracts.

The activity of the renin preparation as determined by the formation of hypertensin was such that an amount corresponding to 0.5 g dry kidney powder gave a maximal yield of hypertensin with globulines from 100 ml horse's serum, when incubated for 10' at 40°. The amount formed corresponded to about 6—10 mg tyramine phosphate in its action on the cat's blood pressure.

### Purification Experiments.

a) Precipitation with saturated ammonium sulphate may give good yields in concentrated renin solutions at pH 4. In a solu-

tion containing the renin obtained from 0.75 g dry powder in one ml the yield was only  $\frac{2}{3}$ . In solutions with the renin from about 4 g dry powder in one ml the yield was constantly good and may be practically 100 %. Such preparations showed a good blood-pressure raising activity in doses of 1 mg pr kg body-weight in cats. With NaCl added to saturation in a solution with the renin from 0.75 g dry powder per ml, about  $\frac{2}{3}$  of the activity was precipitated, the rest being recovered from the filtrate. After the preparation had stood over night in the ice chest, the yield was slightly better. By the addition of NaCl until  $\frac{1}{6}$  saturation at pH 3, inert proteins were precipitated and could be removed. In one experiment, however, only about 50 % of the activity in a concentrated solution was precipitated at saturation (pH 4), and the rest recovered from the filtrate. Ammonium sulphate invariably left no activity in the filtrate from equally concentrated solutions.  $\text{Na}_2\text{SO}_4$  was less effective than  $(\text{H}_4\text{N})_2\text{SO}_4$  and NaCl in precipitating the renin.

b) In another series of experiments, attempts were made to purify the renin by fractionated precipitation with alcohol, acetone and dioxane. The best results were obtained with acetone. To a solution of 1 g crude renin in 20 ml  $\text{H}_2\text{O}$  was added 1 volume of acetone and the precipitate centrifuged off. An addition of a further volume of acetone to the filtrate precipitated some 40 % of the first yield. The combined precipitates were dried with acetone and laked with 10 ml water. A considerable portion of the proteins were insoluble. The clear filtrate was again treated with 1 volume acetone and dried. The weight of this dry preparation was 180 mg. It showed a good activity in 0.2—0.5 mg per kg bodyweight in the cat, as against 1—2 mg in the crude preparation. The purified preparation was readily soluble in water. If the preparation was carried out in a similar way with ethyl alcohol, the yield of activity in the water soluble fraction was less than  $\frac{1}{4}$  of the original. The addition of alcohol up to 30 % caused only little loss, however. With acetone a certain loss was also noted (cf PICKERING and PRINZMETAL, 1938) though it was not so high when the preparation was carried out rapidly and in the cold. In some cases  $\frac{1}{2}$  volume of acetone was added originally which precipitated less than 10 % of the activity but removed a considerable amount of inert proteins. Proteins were also removed by addition of sulphocyanate in a concentration of 0.5 % in watery solution, or by NaCl in 4 % at pH 4.



c) If renin solutions were treated with adsorbents (Fuller's earth, permutite, kaolin or  $\text{Al}_2\text{O}_3$ ), part of the activity was carried down and could be eluted with 0.5 % sodium bicarbonate. When 1 ml of a potent solution of renin acetone dry powder (see above) was treated at pH 4 with 0.2 g of the adsorbents for 1 minute, kaolin was found to be most effective. In further experiments a considerable purification could be obtained by treating purified renin solutions with  $\frac{1}{2}$  their weight of kaolin. Practically no loss was experienced when the kaolin was washed with a 0.1 % solution of acetic acid and the renin eluted with 0.5 %  $\text{NaHCO}_3$ . Of the original activity about 25 % was left in the primary filtrate.

### Formation of a Heat-Stable Dialysable Pressor Substance in Renin Solutions.

To our surprise it was not possible to adsorb the remaining activity in the filtrate after treatment with kaolin. Thus renewed treatment with kaolin left most of the activity in the filtrate. Further observations showed, however, that the active substance was not renin, since the activity in the filtrate withstood heating to  $100^\circ$  for 2' (fig. 1), which renin will not, and

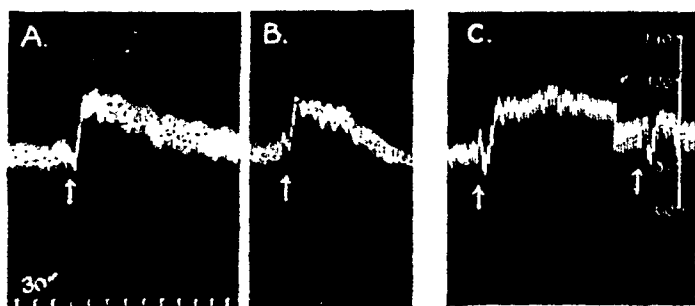


Fig. 1. Cat, chloralose, blood pressure. A. 0.5 ml filtrate from renin solution treated with kaolin. B. Same after heating for 2 min.  $100^\circ$ . C. 0.2 ml kaolin eluate before and after same heat treatment.

was dialysable. The active substance in the filtrate also differed from hypertensin in that it depressed the activity of the isolated rabbit's intestine, much in the same way as the nephren of ENGER (1939, 1941). The considerable amount of this substance found in the filtrate, must have formed gradually in the renin solution, which, when freshly prepared, was inactive after boiling. The

total amount found in the dialysate from 30 ml of the employed renin solution (100 g dry kidney powder) corresponded to no less than 20 mg tyramine phosphate in its action on the cat's blood pressure.

The spontaneous formation of a new pressor substance in dialysed renin solutions evidently constitutes an important source of error in respect of the biological evaluation of renin preparations. A previous paper (EULER and SJÖSTRAND, 1941) referred to this possibility, and we would like to stress this point further in view of our recent experience.

### Summary.

A convenient method for the preparation of renin from pig's kidney is described.

Purification of crude renin preparations has been accomplished by fractionate precipitation with acetone or by adsorption to kaolin.

A dialysable, heat-stable pressor substance may appear spontaneously in dialyzed renin solutions. The new substance has not the properties of hypertensin (angiotonin).

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## Is the Citric Acid Metabolism Affected by Ether Anaesthesia?

By

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In earlier works (MÅRTENSSON, 1938, 1940) it was shown that quantitatively the kidneys play the greatest rôle in the normal mammalian organism for the citric acid (Ci) elimination from the body. The liver deals with far less Ci, especially in the rabbit. Amongst other evidence adduced to show the importance of the kidneys in this respect was the great increase in the citric-acid content of blood serum (Ci/s) after removal of the kidneys. Experiments on animals under urethane anaesthesia revealed that this increase occurred during the hours immediately following the renal extirpation. Experiments were also conducted on animals under ether anaesthesia with a view to following as long as possible, in the waking state, the Ci/s of those animals which survived the nephrectomy. For this purpose only one blood specimen was drawn daily, since more frequent drawings were found to shorten the period of survival very considerably. The tests conducted in this way showed a fall of the Ci/s the day after the operation and thereupon a rise. However, ALWALL found later (1942) high Ci/s values also during the hours immediately following a nephrectomy under ether anaesthesia, i. e. similar conditions under both urethane and ether anaesthesia, which is but natural. He has accordingly furnished further evidence in support of the present writer's view of the importance of the kidneys for Ci-elimination. Actually the Ci/s curve during the first 24 hours after nephrectomy under ether anaesthesia runs the course shown in Fig. 1.

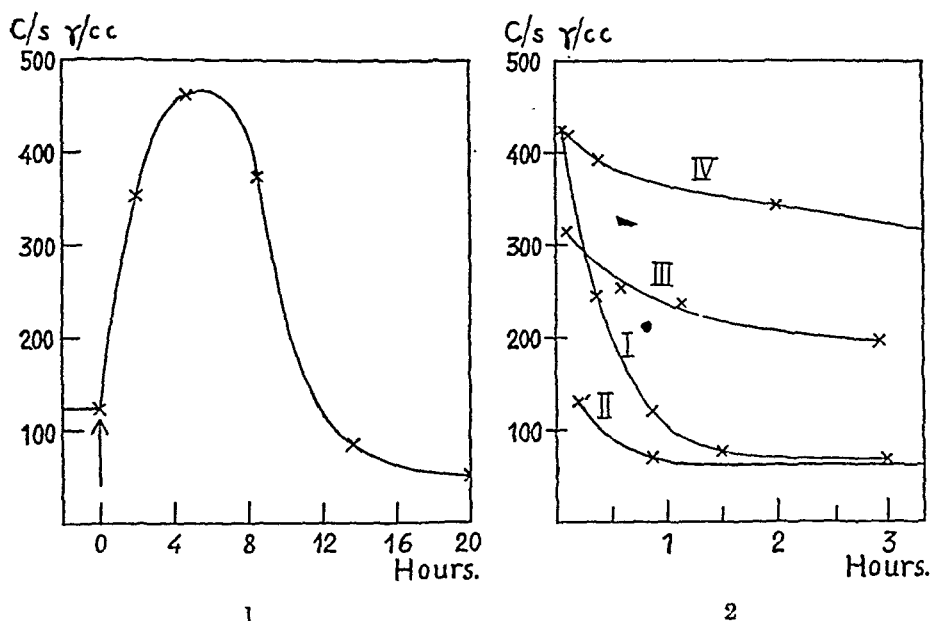


Fig. 1. 16. 10. 42. Rabbit, 2.35 kg.  $\uparrow$  indicates completion of bilateral nephrectomy under ether anaesthesia lasting 20 min. (The ordinate shows, as in the following experiments, the citric-acid content of the blood serum in micrograms per one c.c. The abscissa shows the time in hours.)

Fig. 2. I. 4. 5. 38. Rabbit, 2.5 kg. No anaesthesia. 86 mg Ci/kg intrav.  
 II. 13. 1. 39. Rabbit, 3.9 kg. 44 mg Ci/kg intrav. Nephrectomy under ether anaesthesia 39 hours before.  
 III. 5. 12. 38. Rabbit, 4.7 kg. 36 mg Ci/kg intrav. Nephrectomy under ether anaesthesia 51 hours before.  
 IV. 21. 1. 39. Rabbit, 4.2 kg. 41 mg Ci/kg intrav. Nephrectomy under ether anaesthesia 97 hours before.

In this work the Ci was determined by THUNBERG's enzymatic method. The Ci/s was indicated in the customary way, i. e. in micrograms per one c.c. ( $\gamma/c.c.$ ). Respecting the method reference should be made to the writer's work of 1940.

In a discussion on Ci/s experiments in cases of acidosis the present writer (MÄRTENSSON, 1940) pointed out that the post-operative drop of the Ci/s which had been previously observed in both man (SCHERSTÉN, 1931) and rabbit (GRÖNVALL, 1937) must have another cause than increased renal Ci-elimination, since it also occurs after bilateral nephrectomy. In connexion with this some experiments were also made by injecting definite quantities of Ci intravenously into rabbits from which the kidneys had been removed under ether anaesthesia. These tests showed a considerable Ci-eliminating power the day after the operation, i. e. when

the Ci/s is low, and after that less or no such power (see Fig. 2). At that time the writer had no opportunity of going more closely into the problem of the mechanism underlying the postoperative fall of the Ci/s, and therefore further inquiry into this was deferred. As a tentative explanation, however, it was suggested that the simultaneously appearing acidosis or a possible change in the protein metabolism might be responsible.

However, ALWALL (personal communication) has later been led to assume from similar experiments on nephrectomized rabbits that the present writer's earlier results had been influenced by the fact that the animals had been under anaesthesia and that the liver, which is thought to be especially sensitive to ether anaesthesia, had exhibited a lower Ci-eliminating power than what it actually possesses in a non-anaesthetized state.

It has been previously shown that ether anaesthesia does not alter the Ci/s of normal mammals (a finding also confirmed by ALWALL, 1942). It is *a priori* improbable that the Ci metabolism of the liver should be more sensitive to anaesthesia than the Ci-metabolism of any other organ. Nor is it probable that the very fact of an ether anaesthesia having been undergone will result in the liver acquiring a Ci-eliminating capacity that is larger than the sum of the renal and hepatic capacities before the anaesthesia. Probably other factors play a far greater part in bringing about the postoperative decline of the Ci/s. The following series of experiments on rabbits, it seems to me, is able to give a more direct answer to the question whether ether anaesthesia has any influence on the Ci metabolism.

### Experiments.

A. Under ether anaesthesia both kidneys were extirpated by means of bilateral dorsal section, which was afterwards sutured. The next day, when after its temporary rise the Ci/s had fallen to a subnormal value, ether anaesthesia of the same depth and the same duration as at the operation was again administered. This gave no alteration of the Ci-level.

21. 9. 42. Rabbit, 3.0 kg. Ci/s 53.4  $\gamma$  before and 337  $\gamma$  2 $\frac{2}{3}$  hours after kidney extirpation under ether anaesthesia lasting 25 minutes. The next day the Ci/s was 33.2  $\gamma$  before twenty-five minutes' ether anaesthesia, and 33.6  $\gamma$ , 31.5  $\gamma$ , and 33.0  $\gamma$  respectively 1 hour 10 min., 2 hours and 5 hours after. — Another experiment gave the same result.

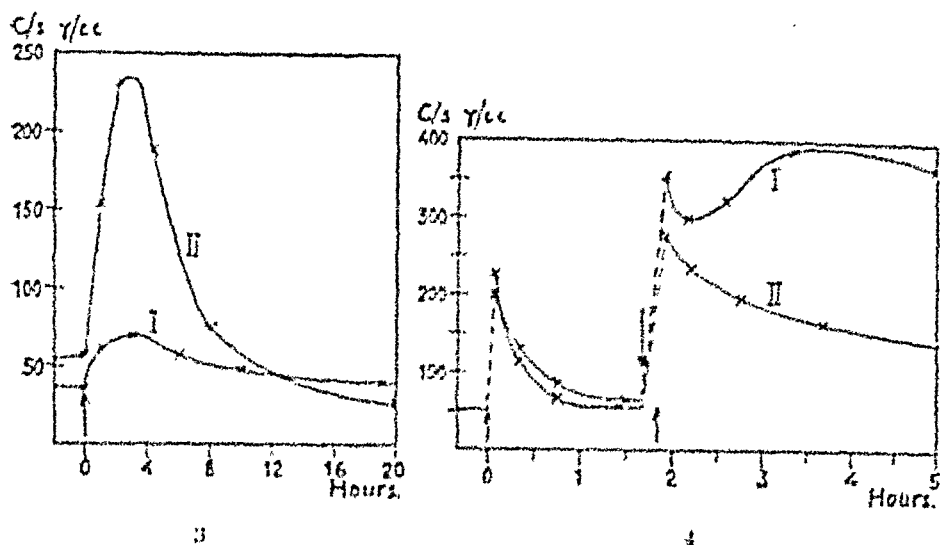
B. The kidneys were removed under ether anaesthesia. The following day the rabbit was given a certain amount of Ci intravenously, and the elimination of the Ci was followed. After  $1\frac{1}{2}$  hours, when the Ci/s had fallen to its initial value, an ether anaesthesia was administered of the same depth but double as long duration as the preceding one, during which Ci injection was repeated. The elimination curve obtained was of the same type both before and after the ether anaesthesia, as can be seen from the following values.

26. 10. 42. Nephrectomized rabbit, 2.4 kg. 5, 20, 45, and 95 minutes after intravenous injection of 60 mg Ci/kg the Ci/s was 218, 105, 55, and 38  $\gamma$  before the ether anaesthesia and 177, 85, 46, and 40  $\gamma$  after.

This experiment also gives an idea of the relatively high Ci-eliminating capacity during the postoperative state, in spite of the absence of the kidneys. Two other experiments arranged in the same way yielded the same result.

C. Under ether anaesthesia an incision was laid dorsally through the skin and abdominal wall to the kidneys, which were not touched however. The cut was sutured. The following day the sutures were removed and the edges of the wound were drawn cautiously apart, whereupon the kidneys could be worked loose and ligatured, to which the animal showed no noteworthy reaction. After this nephrectomy without anaesthesia the Ci/s rose, as after nephrectomy under anaesthesia (see Fig. 3 I). The magnitude of the Ci/s rise when a Ci-eliminating organ is shut off will, it is reasonable to assume, depend upon the quantity of Ci simultaneously formed and the capacity of other Ci-eliminating organs. The rise, in fact, was much sharper when the renal extirpation was not performed until 48 hours after the preparatory operation, i. e. when the Ci/s had come past the postoperative fall and was moving upward (see Fig. 3 II).

D. Under ether anaesthesia a dorsal incision was made in the same manner as in the preceding experiments. The next day the rabbit was given a certain quantity of Ci intravenously, and the elimination was followed. After  $1\frac{1}{2}$  hours, when the Ci/s had dropped to about its initial value, the kidneys were extirpated without anaesthesia and the same Ci administration was repeated immediately. The elimination curve had quite a different appearance after the nephrectomy (see Fig. 4).



- Fig. 3. I. 13. 10. 42. Rabbit, 3.0 kg. Bilateral dorsal section under ether anaesthesia 25 hours earlier. Ci's before this operation 82.5 γ. At  $\frac{1}{2}$  nephrectomy without anaesthesia.  
 II. 30. 10. 42. Rabbit, 2.1 kg. Bilateral dorsal section under ether anaesthesia 46 hours before. Ci's before this operation 97.3 γ, the day after op. 29.6 γ. At  $\frac{1}{2}$  nephrectomy without anaesthesia.
- Fig. 4. I. 3. 10. 42. Rabbit, 2.3 kg. Bilateral dorsal section under ether anaesthesia 25 hours earlier. Ci's before op. 98.3 γ. At  $\frac{1}{2}$  49 mg Ci/kg intrav. Vid  $\frac{1}{2}$  nephrectomy without anaesthesia.  
 II. 26. 10. 42. Rabbit, 2.1 kg. Dorsal section under ether anaesthesia 24 hours earlier. Ci's before op. 89.6 γ. In other respects the same as for I above.

### Discussion.

The writer's earlier experiments tend to indicate that the liver may be the most important Ci-eliminating organ of a nephrectomized animal, although as yet nothing definite is known as to this. As an ether anaesthesia of such an animal does not affect the Ci/s or the Ci-eliminating capacity, this fact ought to constitute rather good evidence against the assumption that the Ci metabolism of the liver is sensitive to ether anaesthesia. That the rise in the Ci/s after a nephrectomy under anaesthesia is not caused by an anaesthetization of the hepatic Ci-eliminating capacity is further shown by the fact that a similar Ci/s rise also occurs after nephrectomy without anaesthesia. The experiments recorded above, therefore, confirm my earlier conclusions that the kidneys of the normal mammalian organism handle the greater portion of the Ci-elimination.

The investigations into the causes of the fall in the Ci/s and the high Ci-eliminating power the day after an operation are being continued.

### Summary.

A series of experiments on rabbits has shown that

1) Ether anaesthesia does not alter the citric-acid content of the blood serum (Ci/s) in a nephrectomized animal, just as little as it does in a normal animal;

2) Ci-elimination in a nephrectomized animal proceeds with the same intensity both before and after an ether anaesthesia;

3) After nephrectomy without anaesthesia the Ci/s rises, as it also does after nephrectomy under anaesthesia;

4) After nephrectomy without anaesthesia the elimination of administered Ci is considerably slowed up as compared with the elimination before the nephrectomy.

The experiments show that the anaesthesia administered in the writer's earlier animal experiments did not influence the results of the investigation, which have accordingly been confirmed.

The present investigation has been conducted with the assistance of a grant from "Stiftelsen Therese och Johan Anderssons Minne"

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## Borsäure und Standardstoffwechsel.

Von

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Eingereicht am 24. November 1942.

Bor ist ein normaler Körperbestandteil des tierischen Organismus (BERTRAND und AGULHON 1912, 1913, HOVE, ELVEHJEM und HART 1939). Während es aber bei Pflanzen für Wachstum und normale Entwicklung notwendig ist, dürfte ihm bei Tieren keine ähnliche Bedeutung zukommen. Hier ist es wahrscheinlich nur als zufällige Verunreinigung zu betrachten, die durch den Borgehalt der Nahrung verursacht wird. HOVE, ELVEHJEM und HART fanden nämlich, dass das Wachstum von Ratten, die mit der Kost nur 0.8 Mikrogramm Bor pro Tag erhielten, normal war, was ORENT-KEILES (1940) bestätigt hat. Wurde mehr Bor zugeführt, wurde das Wachstum nicht gebessert. Andererseits wurde eine Abnahme des Reproduktionsvermögens bei weiblichen Ratten beobachtet, wenn die tägliche Boreinnahme auf 1 000 Mikrogrammen stieg. PUHLMANN (1939) hat ebenfalls bei genügenden Mengen eine Beeinflussung der Sexualfunktionen gesehen. Bei weiblichen Ratten, die während 10 Tage mit dem Futter 0.5 Proz. Borsäure verzehrten, trat eine reversible Brunsthemmung ein. Eine ähnliche Brunsthemmung oder Dauerbrunst sah er nach täglichen subkutanen Injektionen von 0.75—1.0 g pro kg.

Auch beim Menschen können u. U. Giftwirkungen von der Borsäure hervorgerufen werden; sogar Todesfälle infolge subkutaner Einverleibung von Borsäurelösung (durch Verwechslung mit physiologischer Kochsalzlösung) sind beschrieben worden (CUSHING 1930, VARTIAINEN und ORAVAINEN 1933). Es geht aber auch aus diesen Beobachtungen hervor, dass die Giftigkeit nicht besonders gross ist.

Sehr umstritten ist dagegen, inwieweit mässige Borsäuremengen bei peroraler Zufuhr ebenfalls ungünstig einwirken. Diese Frage ist von Interesse teils wegen der ab und zu empfohlenen Anwendung der Borsäure als therapeutisches Mittel, teils und hauptsächlich aus dem Grunde, dass Borsäure eine wichtige Rolle zum Konservieren von Nahrungsmitteln gespielt hat und in gewissen Fällen (für Krabben, Kaviar, Eigelb) noch spielt. Unter den zahlreichen Arbeiten hierüber sind die umfassenden Untersuchungen von ROST und seinen Mitarbeitern (1903) besonders bekannt. Sie haben zweifelsohne zu den Einschränkungen in bezug auf die Verwendung der Borsäure als Konservierungsmittel in vielen Ländern Anlass gegeben. Aus den Untersuchungen ging hervor, dass lokale Reizerscheinungen (Rötung, Entzündung, Ätzung) nur bei sehr grossen Dosen und hohen Konzentrationen entstehen und zwar vorzugsweise bei Versuchstieren, die sich nicht durch Erbrechen schützen können. Die vom Menschen eingenommene Borsäure wird ziemlich langsam eliminiert, so dass eine gewisse Gefahr der Kumulation im Körper bestehen soll. ROST behauptet, Wirkungen auf die Ausnutzung der Kost und auf den Stoffwechsel gefunden zu haben. Nach 1—3 g Borsäure täglich soll eine Herabsetzung der Ausnutzung eintreten. Was den Stoffwechsel betrifft, beobachtete ROST nach täglicher Zufuhr von 3 g Borsäure während 12 Tage (7 Versuche an 5 Personen) einen Gewichtsverlust, der seiner Meinung nach nicht vollständig von der durch die Borsäure hervorgerufenen Diuresesteigerung erklärt werden kann. ROST meint, dass die Borsäure eine Vermehrung der Fettverbrennung bewirkt hat. Damit schien zu stimmen, dass RUBNER (1903) bei zwei der Versuchspersonen Vermehrung von Wasser- und Kohlendioxydabgabe bei relativer Ruhe während der Borsäureperiode im Vergleich mit der Normalperiode beobachtete.

Die Untersuchungen von ROST und RUBNER wurden von HANSEN und GLOPPE (1932) eingehend kritisiert. Bezüglich der gefundenen Herabsetzung der Ausnutzung der Kost heben sie hervor, dass der Befund unregelmässig ist — er wird in gewissen älteren Versuchen aber auch bei einigen von ROSTS Versuchspersonen vermisst. Ob die Erklärung in eine schwach abführende Wirkung oder in einen spezifischen Effekt der Borsäure gesucht werden soll, ist unbekannt. Bei Versuchspersonen, die auf gewöhnlicher, nicht begrenzter Kost stehen, ist diese Wirkung praktisch ohne Bedeutung, während sie bei knapper Kost vermutlich zu

Bezeichnungen: 1= Zahl der Tage, 2= Zahl der Tage, an welchen Bestimmungen gemacht

Versuchs- person	V o r p e r i o d e				B o r s ä u r e p e r i o d e <sup>1</sup>			
	1	2	3	4	1	2	3	4
A. A. 81 kg. . .	11	10	292.2 ± 6.30	98.4 ± 1.62	11	10	266.0 ± 5.96	91.0 ± 1.81
H. J. 70 kg. . .	11	10	259.1 ± 2.64	101.0 ± 1.47	11	10	243.3 ± 3.05	96.4 ± 0.87
S. W. 68 kg. . .	22	19	222.3 ± 1.47	93.3 ± 0.72	5 <sup>2</sup> 16 <sup>1</sup>	5 14	217.2 ± 1.92 217.5 ± 1.54	91.4 ± 0.99 92.4 ± 0.64
E. B. 70 kg. . .	14	12	231.9 ± 4.04	96.1 ± 0.96	15	12	223.8 ± 2.96	92.5 ± 0.81

einer Abmagerung beitragen kann. Von gewisser Bedeutung ist dabei auch die vermehrte Diurese und Wasserabgabe auf anderen Wegen. Die von RUBNER beobachtete Steigerung des Stoffwechsels ist nicht unter Standardbedingungen gefunden worden, sie kann deshalb sehr wohl indirekter Natur sein. Ehe Bestimmungen unter wirklichen Ruhebedingungen existieren, ruht die ganze Lehre von der spezifischen Wirkung der Borsäurepräparate auf den Stoffwechsel auf Vermutungen.

Die Einwendungen von HANSEN und GLOPPE sind unserer Meinung nach vollkommen berechtigt. Es ist unzweifelhaft, dass die von RUBNER gefundene Erhöhung der Verbrennungsprozesse nicht für eine spezifische Wirkung der Borsäure beweisend ist. Die nähere Durchsicht der Versuche zeigt dies sehr deutlich. Die Kohlendioxydabgabe während der Normalperioden betrug bei seinen Versuchspersonen pro 24 Stunden im Durchschnitt 678.2 bzw 692.9 g. Hieraus ergibt sich eine CO<sub>2</sub>-abgabe pro Minute von 240, bzw 245 ml. Nimmt man einen respiratorischen Quotienten von 0.75 an, erhält man einen Sauerstoffverbrauch von 320, bzw 326 ml pro Min., was (bei dem Körpergewicht von 63, bzw. 59 kg) entschieden beweist, dass wirkliche Ruhebedingungen nicht vorhanden waren. Dies erklärt auch die sehr erheblichen Schwankungen von Tag zu Tag. Berechnet man in gewöhnlicher Weise den mittleren Fehler des Mittels, erhält man für RUBNERS Versuchspersonen die folgenden Werte für die Kohlendioxydabgabe für 24 Stunden (in g):

<sup>1</sup> 3 g Borsäure pro Tag.

<sup>2</sup> 0.5 g Borsäure pro Tag.

belle.

### Standardstoffwechsels.

wurden, 3= O<sub>2</sub>-Verbrauch, ml pro Min., 4= Relativer Standardstoffwechsel in Prozent.

Nachperiode				Differenz Vorperiode— Borsäureperiode		Differenz Borsäureperiode —Nachperiode	
1	2	3	4	3	4	3	4
12	10	269.8 ± 2.78	90.6 ± 0.96	26.2 ± 8.67	7.4 ± 2.43	- 3.8 ± 6.56	0.4 ± 2.05
12	10	248.1 ± 1.94	97.7 ± 0.85	15.8 ± 4.03	4.6 ± 1.71	- 4.8 ± 3.62	- 1.3 ± 1.21
				5.1 ± 2.48	1.9 ± 1.22		
4	4	218.8 ± 3.26	93.0 ± 1.34	4.8 ± 2.13	0.9 ± 0.96	- 1.3 ± 3.60	- 0.6 ± 1.48
13	11	223.1 ± 1.72	92.5 ± 0.94	8.1 ± 5.20	3.6 ± 1.25	0.7 ± 3.42	0.0 ± 1.24

Versuchs- person	Normalperiode	Borsäureperiode (3 g Borsäure täglich)	Differenz
Al . . . .	678.2 ± 11.7	795.2 ± 43.1	117.0 ± 45.0
Br . . . .	692.9 ± 8.6	738.6 ± 22.0	45.7 ± 23.6

Die Differenzen sind offenbar nicht statistisch sichergestellt.

In Unseren Versuchen wurden der Einfluss der Borsäure auf den Standardstoffwechsel (Grundumsatz) bei vier gesunden männlichen Versuchspersonen im Alter von 25—30 Jahren bestimmt. Die Versuchsperson kam täglich — mit Ausnahme der Sonntage — früh am Morgen nüchtern ins Laboratorium, ruhte etwa  $\frac{3}{4}$  Stunden bequem in einem Leigestuhl, worauf der Sauerstoffverbrauch mit dem Spirometer von KROGH (1920) ermittelt wurde. Bei den zwei ersten Versuchspersonen (A. A. und H. J.) wurde nur eine tägliche Bestimmung ausgeführt, bei den zwei später untersuchten (S. W. und E. B.) wurden dagegen regelmässig zwei Bestimmungen kurz nach einander gemacht, aus denen dann der Mittelwert des betreffenden Tages erhalten wurde. Für jede Versuchsperson kam zuerst eine Vorperiode, die bei A. A. und H. J. 11 Tage (mit 10 Bestimmungen) umfasste, bei E. B. auf 14 Tage (12 Doppelbestimmungen) und bei S. W. auf 22 Tage (19 Doppelbestimmungen) ausgedehnt wurde. Unmittelbar nach der letzten Bestimmung der Vorperiode wurde Borsäure gegeben und zwar 1 g 3 mal täglich in Kapseln nach dem Essen. Am folgenden Tage wurden mit neuen Bestimmungen des Sauerstoffverbrauchs angefangen. Die Borsäureperiode dauerte 11 bis 16 Tage; in einem Falle (bei S. W.) wurde zuerst nur 0.5 g Borsäure täglich während 5 Tage gegeben, dann kamen 16 Tage mit 3 g Borsäure pro Tag. Endlich folgte bei sämtlichen eine Nachperiode von 4 bis 13 Tagen ohne Borsäure. Aus den gefundenen Werten für den Sauerstoffverbrauch wurde teils das Mittel der betreffenden Periode nebst dessen mittleren Fehler in gewöhnlicher Weise berechnet, teils wurde auch unter Beachtung des täglich festgestellten

Körpergewichtes und der Körperlänge sowie Alter der Sauerstoffverbrauch nach HARRIS und BENEDICT (1919) ermittelt. In der Tabelle oben wird das Verhältnis zwischen dem direkt gefundenen Werte und dem nach BENEDICT und HARRIS berechneten als relativer Standardstoffwechsel bezeichnet.

Die Versuchspersonen hatten von der Borsäure keine Unbehagen; nur in einem Falle spürte die Versuchsperson nach 4 Tagen leichte Bauchschmerzen, die bald vorübergingen. Das Körpergewicht hielt sich in allen Fällen mit kleinen Schwankungen in beiden Richtungen annähernd konstant.

Unsere Ergebnisse werden in der Tabelle zusammengestellt; um Raum zu ersparen, werden nur die Mittelwerte für die betreffenden Perioden mit ihren mittleren Fehlern angegeben.

Die Werte liegen so gut wie immer etwas niedriger als der Standard von HARRIS und BENEDICT. Dies steht in guter Übereinstimmung mit früheren Ergebnissen aus Schweden, indem bei genügend langer Ruhe vor dem Versuch nach mehreren Verfassern ähnliche Abweichungen nach unten gefunden wurden (z. B. LILJESTRAND und STENSTRÖM 1925, WISING 1934).

Ein Blick auf die Tabelle zeigt sofort, dass die Zufuhr der Borsäure keine Erhöhung des Sauerstoffverbrauchs hervorgerufen hat. Aus den Differenzen zwischen den Mittelwerten der Vorperiode und der Borsäureperiode einerseits und zwischen der Borsäureperiode und der Nachperiode andererseits, wie sie in den beiden letzten Spalten der Tabelle gegeben werden, ist ersichtlich, dass der Sauerstoffverbrauch in der Borsäureperiode in keinem Falle grösser als in der Vor-, bzw. der Nachperiode ist. Überhaupt zeigt sich für jede Versuchsperson eine auffallende Konstanz der Sauerstoffwerte während des ganzen Versuchs. In zwei Fällen (A. A. und H. J.) ist der Sauerstoffverbrauch in der Vorperiode sehr wenig höher als in den beiden späteren; wenn die kleinen Schwankungen des Körpergewichtes berücksichtigt werden, wie es bei der Berechnung des relativen Standardstoffwechsels der Fall ist, sinkt bei H. J. der Unterschied unter 3 mal des mittleren Fehlers und ist also nicht statistisch sicher, während er bei A. A. eben als sicher betrachtet werden kann. Vermutlich bedingt die zunehmende Übung im Laufe des Versuchs eine vollständigere Muskelruhe, wodurch der Unterschied erklärt werden dürfte. Aus dieser Überlegung wurden bei S. W. und E. B. die Vorperioden etwas länger genommen, und jetzt fallen alle Differenzen innerhalb der Fehlergrenzen. Wir meinen deshalb, dass wir berechtigt

sind, aus unseren Versuchen die Schlussfolgerung zu ziehen, dass 3 g Borsäure täglich genommen während 10—16 Tage beim erwachsenen Menschen den Ruhestoffwechsel nicht ändert. Die Behauptung, dass die Borsäure in diesen Dosen einen ungünstigen Einfluss auf den Stoffwechsel ausübt, kann nicht aufrechterhalten werden, und die Auffassung, dass die erwähnten Mengen von Borsäure schädlich wirken, muss deshalb revidiert werden.

### Zusammenfassung.

Bei vier gesunden männlichen Versuchspersonen hatte 3 g Borsäure, täglich während 11—16 Tage genommen, keinen Einfluss auf den Standardstoffwechsel.

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## The Absorption of Calcium as a Function of the Body Saturation with Calcium.

By

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ROTTENSTEN (1938) found that young rats previously fed on a calcium rich diet absorbed less calcium than rats previously on a diet poor in calcium, when the absorption was tested in the two groups with the same amount of calcium in the diet.

Children on diets rich in calcium and vitamin D absorb considerable amounts of calcium, as compared with adults. The same phenomenon can be observed in rats.

The experiments here presented represent a more detailed study of the mechanism concerned.

### Experimental.

Only one calcium "free" basal diet was used throughout. It consisted of:

10 %	wheat gluten
2 %	egg albumin
3 %	dried brewer's yeast
84 %	whole wheat flour
1 %	NaCl
0.2 %	ferrous citrate

with a calcium content of 0.04 %.

To this diet were added different amounts of calcium carbonate and calcium phosphate according to the planning of the actual experiment. Vitamin A was supplied as "Vogan" 10 I. U. daily. Only two series of experiments were performed in vitamin D deficient rats. In all the other experiments 20 I. U. vitamin D<sub>2</sub> was given daily.

# The Absorption of Calcium in Young Rats a) with and b) without Vitamin D, and on Different Levels of Calcium in the Diet.

## Experiment a.

Two groups of rats, four weeks old were fed on the basal diet for 1 month, one group receiving  $20 \pm 1$  mg Ca in the diet (0.4 %  $\text{CaCO}_3$  in the diet), the other group about 100 mg Ca daily (1 %  $\text{CaCO}_3$  and 1 %  $\text{CaHPO}_4$  in the diet).

Next absorption experiments were started (technique see NICOLAYSEN and JANSEN 1939). The results are given in Table I.

Table I.

*Ca rich versus Ca poor diet; 20 I. U. vitamin D daily.*

Previously 0.95 % Ca in the diet		Previously 0.2 % Ca in the diet	
5th week	6th week	5th week	6th week
mg Ca absorbed daily (Ca intake about 100 mg daily)			
14	19	30	26
20	20	31	26
21	21	32	27
21	21	33	30
21	22	38	30
22	24	39	30
22	25	39	34
25	26	40	35
26	26	40	36
28	27	41	37
28	28	42	41
31	29	49	42
Average: 23	24	38	33
% absorption 11—22	15—26	25—36	20—29

The rats previously fed on a calcium poor diet absorb distinctly more calcium when shifted over to a Ca rich diet, than those previously fed on a Ca rich diet.

The difference is more pronounced in the 5th week of the experiment *i. e.* in the first absorption experiment than in the following week.

The rats continuously on a diet rich in calcium absorbed less calcium in the later than in the earlier weeks. Taking the experiments later reported into account, this result demonstrates how rapidly the high absorption ability may decrease.



The chief inference to be drawn from these observations is that there is an endogenous factor in the regulation of the absorption of calcium.

### Experiment b

was a true repetition of *exp. a* with the only difference that the rats now received no vitamin D. Table II gives the results. The ability to absorb calcium is low in both groups of rats, and there is a statistically hardly significant difference between the two groups.

Table II.

*Ca rich versus Ca poor diet — vitamin D.*

Previously 0.95 % Ca in the diet		Previously 0.2 % Ca in the diet	
5th week	6th week	5th week	6th week
mg Ca absorbed daily (Ca intake about 75 mg daily)			
	4		
8	5		
10	8	11	4
10	8	11	5
12	8	12	7
13	9	13	9
13	10	14	10
14	11	15	11
14	11	15	12
14	12	17	13
15	13	17	15
15	15	21	18
Average: 12.5		8.7	14.6
			10.4

This result indicates that although the saturation of the body with calcium influences the ability to absorb calcium, vitamin D is the prime regulating factor, without which the endogenous factor loses practically all effect.

### The Absorption of Calcium from Isolated Loops of the Small Intestine in Rats on Different Levels of Calcium in the Diet.

These experiments were a true repetition of experiments a) above reported, with the one difference that now the absorption after one month was studied from isolated loops.

The technique has been described in an earlier paper (NICOLAYSEN 1937). The results are given in Table III.

Table III.

*Absorption of Ca from isolated loops of the jejunum. 3 ml  $\text{CaCl}_2$  containing 10 mg Ca injected into 30 cm loops.*

Previously 0.95 % Ca in the diet 20 I. U. vitamin D daily	Previously 0.2 % Ca in the diet 20 I. U. vitamin D daily
mg Ca absorbed in 5 hours	
1.9	
2.2	
2.7	3.4
2.8	4.2
2.9	4.5
3.5	4.8
3.5	5.0
3.6	5.1
3.7	5.1
3.8	5.5
4.7	6.5
Average: 3.2	4.9

It appears that the endogenous factor influences the velocity with which calcium passes through the intestinal epithelium. When plotted graphically, these results and the results obtained in experiment a) give curves of essentially the same type, although the spreading of the results is greater in the last experiments. There is always an uncertainty in the measuring of a 30 cm loop, this may be a sufficient explanation of the greater spreading. It is logical to conclude, however, that the endogenous factor acts by increasing the velocity with which calcium passes through the intestinal epithelium.

### Experiments in Old Rats.

A group of two year old rats were given the calcium "free" basal diet + 20 I. U. vitamin D daily for 4 months. Next the absorption of Ca was studied in ordinary weekly metabolism experiments as well as from isolated loops.

The results are given in Tables IV and V from which it is seen that the absorption of calcium is very slow as compared with the absorption in young rats on a Ca poor diet. The ash content of

the bones in the old rats was about 55 % as compared with about 65 % in adult not Ca starved rats.

Table IV.

*Absorption of Ca in 2 year old rats previously Ca starved for 4 months. 20 I. U. vitamin D daily.*

Daily intake about 30 mg Ca.	
Old rats	2 month old rats, 0.2 % Ca in the diet
mg Ca absorbed daily	
	25
4	26
4	22
6	22
8	27
10	27
13	25

Table V.

*Ca absorption from isolated loops of the jejunum in 2 year old rats previously for 4 months on a Ca free diet. 3 ml CaCl<sub>2</sub> containing 10 mg Ca injected into 30 cm loops.*

10 weeks old rats, previously for 6 weeks on 0.2 % Ca in the diet	old rats
mg Ca absorbed in 5 hours	
5.8	
5.9	
6.0	1.9
6.1	2.3
6.1	2.3
6.3	2.4
6.8	2.5
6.9	2.9
7.4	2.9

### Experiments in Pregnant Rats.

A group of female rats about 1 year old were put in a breeding cage together with a suitable amount of bucks. They were all given the calcium "free" basal diet + 20 I. U. vitamin D daily.

When the females 2-3 weeks later were observed to be pregnant they were isolated in separate cages. A series of experiments were then performed on them either the day after parturition or just before (the fetuses removed by Cesarean section thrive just as well as those born the natural way).

Table VI gives the results which demonstrate that the absorption is just as slow as in old rats. The endogenous factor thus does not influence calcium absorption in pregnancy.

Table VI.

*Absorption of Ca from isolated loops of the jejunum. 3 ml  $\text{CaCl}_2$  containing 10 mg Ca injected into 30 cm loops in pregnant rats at term.*

2-month old rats 0.2 % Ca in the diet for 1 month 20 I. U. vitamin D daily mg Ca absorbed in 5 hours	Pregnant rats Ca starved 20 I. U. vitamin D daily
3.4	
4.2	
4.5	2.2
4.8	2.3
5.0	2.4
5.1	2.6
5.1	2.6
5.5	2.9
6.5	4.3
Average: 4.9	2.8

### Calcium Absorption and the Endocrine Glands: Thymus and the Gonads.

As the endogenous factor apparently disappeared at a young age, it might be that the two endocrine glands undergoing essential functional changes at the time of puberty, had something to do with the regulation of the absorption of calcium.

Young female rats were given the basal diet with the addition of 0.4 %  $\text{CaCO}_3$  and 20 I. U. vitamin D daily. Vaginal smears were taken at intervals until oestrus appeared. The absorption of Ca was then studied from isolated loops. Table VII gives the results. The speed of absorption is the same in sexually mature rats as in younger, still sexually immature rats fed for 4 weeks on the same Ca poor diet.

A group of 4 week old rats were thymectomized.

The sternum was cut longitudinally down to over the heart and thymus removed whole in its capsule. Extraordinarily light blunt dissection is necessary in order not to severe pleura. Of 28 rats 6 died

of phenumothorax, the rest survived and thrive just as well as young non-operated rats. The operation which takes 4—5 minutes was performed in ether anesthesia.

Table VII.

*Absorption of Ca from isolated loops of the jejunum. 3 ml CaCl<sub>2</sub> containing 10 mg Ca injected into 30 cm loops in sexually mature female rats.*

2 month old rats 0.2 % Ca in the diet for 1 month 20 I. U. vitamin D daily	Previously 0.2 % Ca in the diet, rats in oestrus 20 I. U. vitamin D daily
mg Ca absorbed in 5 hours	
3.4	
4.2	
4.5	
4.8	3.2
5.0	3.7
5.1	4.1
5.1	4.9
5.5	5.2
6.5	5.2

After 4 weeks on the diet with 0.4 % CaCO<sub>3</sub> + 20 I. U. vitamin D daily the absorption was studied in isolated loops.

From Table VIII it is seen that the absorption is just as rapid as in non-operated rats on a Ca poor diet and distinctly quicker than in a group of rats previously fed on a Ca rich diet.

Table VIII.

*Absorption of Ca in 2 month old rats 1 month after thymectomy. 20 I. U. vitamin D daily. 3 ml CaCl<sub>2</sub> containing 10 mg Ca injected into 30 cm loops of the jejunum.*

Not operated rats		Thymectomized rats
Previously 0.95 % Ca in the diet	Previously 0.2 % Ca in the diet	Previously 0.2 % Ca in the diet
mg Ca absorbed in 5 hours		
	3.4	
	4.2	
2.7	4.5	3.1
2.8	4.8	3.4
3.5	5.0	3.6
3.7	5.1	4.4
3.7	5.1	5.2
3.7	5.5	5.6
4.3	6.5	5.6

## A Detailed Study of the Calcium Absorption in Young Rats.

Here three different series of experiments will be reported.

1. The rats used in the first experiments were fed from 4 weeks of age as follows:

For 4 weeks about 20 mg Ca in the daily ration,

for 5 weeks (in the first two the absorption was studied as reported in experiment a)) about 100 mg Ca in the daily ration,

for 3 weeks about 30 mg Ca daily,

for 9 weeks about 5 mg Ca daily.

The rats were then 6 months old. The absorption was studied at this age and when on about 30 mg Ca daily. Table IX gives the results.

Table IX.

*Ca absorption in rats at 3½ and 6 months of age after different levels of Ca in the diet 20 I. U. vitamin D daily. About 30 mg Ca in the daily ration.*

3½ months old after 5 weeks on 0.95 % Ca in the diet	6 months old after 9 weeks on a Ca >free diet
mg Ca absorbed daily	
7	
9	
10	4
11	7
12	7
12	8
13	8
13	8
13	8
14	9
15	9
15	10
<hr/>	
Average: 12 mg	8 mg
% absorption: 36	26

The percentage Ca absorbed is very low as compared with the percentage absorption in rats fed on a Ca poor diet continuously for 6 months (see the next experiment).

2. A group of 12 rats was taken into experiment when 4 weeks old. Every month absorption was tested in weekly experiments with 0.4 % CaCO<sub>3</sub> added to the diet. In the intervals they were fed on decreasing amounts of Ca (0.11 % Ca in the first 3 months;

0.07 % Ca in the following months). When 6 months old the rats were given 1 %  $\text{CaCO}_3$  + 1 %  $\text{CaHPO}_4$  in the diet for 4 weeks. Finally the absorption was again studied with 0.25 % in the diet.

Figure 1 summarizes the results. It is seen that the absorption drops from over 80 to near 60 % in the first months, but

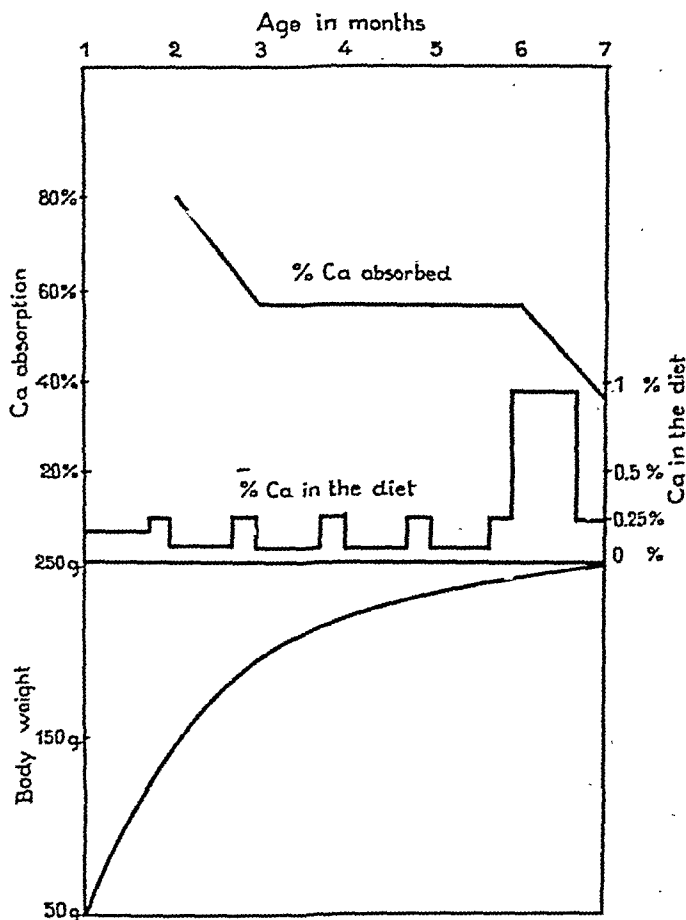


Fig. 1.

that it is then maintained at this level, until a Ca rich diet provokes a distinct fall again. The Ca intake in these experiments was about 30 mg daily in the first months and then increased to between 30 and 40 mg daily.

3. A group of 12 rats was fed on a diet with 1 %  $\text{CaCO}_3$  + 1 %  $\text{CaHPO}_4$  for 8 months without any vitamin D. Next they were given 20 I. U. vitamin D daily and the absorption studied in one week's experiment on 0.25 % Ca in the diet.

Whenever changing from a Ca rich to a Ca poor diet before absorption experiments, the rats were for 3 days given the diet with 0.25 % before the absorption experiment was started.

The rats were again put on the Ca rich diet for 1 month after which the absorption was again tested on 0.25 % Ca in the diet. The results are given in Table X.

Table X.

*Ca absorption in rats fed on a vitamin D free Ca (0.95 %) and P rich diet for 8 months, and then given 20 I. U. vitamin D daily.*

mg Ca daily in the food	% absorption
31	78
32	78
37	76
37	73
38	74
41	73
44	64
45	66
45	78
48	77
49	74
49	73

*Ca absorption in the same rats 1 months later, after 0.95 % Ca and 20 I. U. vitamin D in the diet.*

mg Ca daily in the diet	% absorption
25	28
33	21
34	27
36	20
37	24
38	24
42	26
43	26
46	22
46	26
46	28
50	26

The 8 month old rats demonstrate a highly efficient absorption of Ca when given vitamin D, but the absorption rapidly drops to the adult level when the body is saturated with Ca by the aid of Ca and vitamin D in the diet.



## Discussion.

The three last series of experiments are without any details reproduced in Fig. 2.

The essential points are: 1. When the body is unsaturated with calcium at that age when growth slows down, Ca starvation cannot again increase the velocity with which Ca passes through the

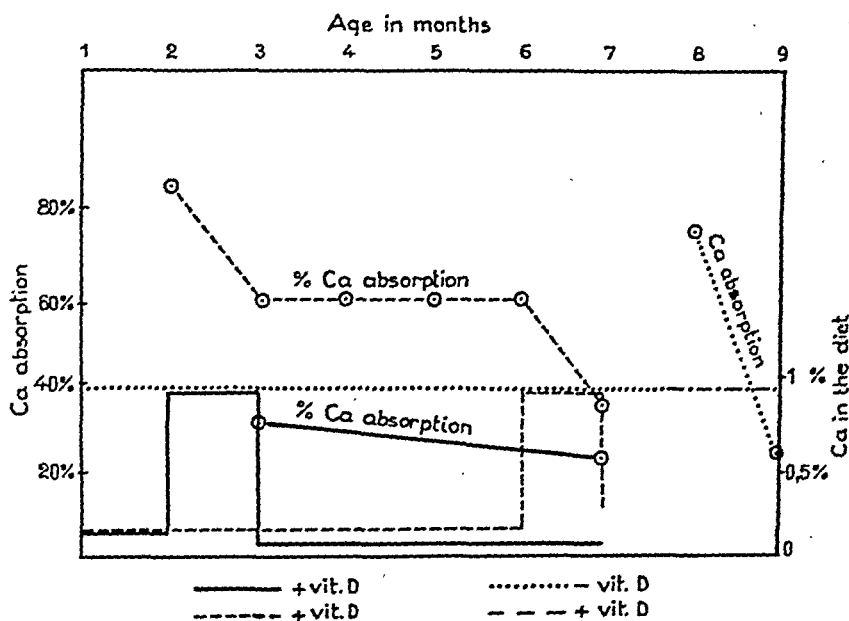


Fig. 2.

intestinal epithelium. 2. When the body is kept unsaturated with calcium, the velocity of Ca absorption may be kept on a very high level until adult age.

All experiments taken into account it is hardly possible, that the different levels of Ca in the diet affect the intestinal epithelium by a direct effect on this membrane. The first of the last three experiments points strongly against such an interpretation.

Ca starvation in adults provokes osteoporosis and not any rich recurrence of osteoid tissue. In a skeleton continuously undernourished with Ca from a very early age, osteoid tissue will continue to be present until saturation with bone salts.

Rats fed on a Ca and P rich diet without any vitamin D may calcify their skeleton very well, still there is an essential difference in the architecture of the bones as compared with rats

on the same diet + vitamin D. (NICOLAYSEN and JANSEN 1939). The difference rapidly disappears on addition of vitamin D.

The logical interpretation of the endogenous regulation of Ca absorption, as described in this paper, is that the immature skeleton, possibly by the production of a hormone, influences the speed with which Ca passes through the intestinal epithelium. However, it would be premature more than to merely suggest such an explanation.

### Summary.

1. The speed of absorption of calcium in rats is influenced by the levels of calcium in the diet; but only as long as the skeleton is kept unsaturated with calcium salts from an early age.

2. Calcium starvation in adult rats and in pregnancy does not increase the speed of absorption of calcium.

3. Thymus and the gonads do not appear to influence the absorption of Ca.

### Conclusion.

There is in growing animals an endogenous factor in the regulation of calcium absorption. This factor has hardly any effect in the absence of vitamin D, which consequently in growing animals is the prime regulating factor. In adults vitamin D exerts only little effect on the absorption of calcium because the endogenous factor is absent.

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## Calcium metabolism and citric acid.

By

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SHOHL (1937) reported that citric acid-citrate mixture added to the diet prevented rickets in rats. As citric acid forms a soluble complex calcium salt, the explanation suggested itself, that Ca and P absorption was promoted.

DICKENS (1941) found rich amounts of citric acid in the skeleton. In the bones of a rachitic kitten the concentration was reduced by 50 %. Now vitamin D and the endogenous factor in calcium absorption (NICOLAYSEN 1943) act analogously on the passage of calcium through the intestinal epithelium. It might be that they both acted through the citric acid, which could be thought to transport Ca through the intestinal wall.

### Experimental.

First an attempt was made to cure rickets in rats by subcutaneous injections of sodium citrate, but even very large doses were without any effect at all. It was next planned to study the absorption of calcium citrate from isolated loops. However, this salt is not soluble enough to permit reliable studies of Ca absorption with the technique used in such experiments.

As citric acid injected or ingested is nearly completely combusted in the body, these experiments have not completely ruled out the suggested hypothesis.

## The Citric Acid in the Skeleton as Influenced by Vitamin D and Different Levels of Calcium in the Diet.

A group of rats was given a rachitic diet: 5 % egg albumin, 1 % NaCl, 1 % tinned spinach, 2 %  $\text{CaCO}_3$ , 91 % patent flour (on this diet severe rickets is constantly produced in this laboratory in 15 days). Another group was given the same diet but with 1 %  $\text{CaCO}_3$  + 1 %  $\text{CaHPO}_4$  and 20 I. U. vitamin D daily to each rat. After 15 days the rats were killed. The citric acid (pentabromacetone method) and the ash content of the bones were analysed.

Next two groups of rats were given the Ca "free" basal diet used in the previous publication. One group received 1 %  $\text{CaCO}_3$  + 1 %  $\text{CaHPO}_4$ , the other 0.4 %  $\text{CaCO}_3$  in the diet. All rats were given 20 I. U. vitamin D daily. After 1 month the rats were killed and citric acid and the ash content of the bones were estimated.

The results are given in the tables 1 and 2.

Table 1.

### *Citric acid in the skeleton.*

+ vitamin D Ca and P rich diet			- vitamin D Ca rich P poor diet		
Citric acid %	ash %	6—7 week old rats	Citric acid %	ash %	
		<u>ash</u> citric acid			<u>ash</u> citric acid
0.66	49.2	85	0.14	25.5	175
0.66	49.2	85	0.12	30.8	260
0.50	41.1	80	0.13	27.0	210
0.56	48.4	85	0.16	28.2	180

Table 2.

### *Calcium rich versus calcium poor food.*

+ vitamin D

Ca rich food 0.95 % Ca in the diet			Ca poor food 0.2 % Ca in the diet		
Citric acid %	ash %	9 week old rats	Citric acid %	ash %	
		<u>ash</u> citric acid			<u>ash</u> citric acid
			0.46	46.4	101
0.68	55.0	80	0.49	43.9	89
0.50	56.5	112	0.42	43.9	106
0.53	57.5	109	0.52	45.4	87
0.60	60.3	101	0.42	44.2	105

The citric acid content of the bones in Ca subnourished rats is lower than in the Ca rich rats, but the ratio  $\frac{\text{ash}}{\text{citric acid}}$  is the same in the two groups.

The vitamin D deficient rats present quite another picture. Here not only is the citric acid depressed to about  $\frac{1}{5}$  of the normal content, but the ratio  $\frac{\text{ash}}{\text{citric acid}}$  is doubled. Thus vitamin D influences the concentration of citric acid in the bones independent of the calcification.

These experiments indicate, that vitamin D and the endogenous factor in the skeleton may promote Ca absorption in different ways.

### Summary.

Injections of citric acid will not cure rickets in rats. Ca subnourishment depresses the citric acid content of the bones but only in proportion to the reduction of the ash content.

In vitamin D deficient rats the citric acid content of the bones is greatly reduced, but in disproportion to the decrease of the ash content. The ratio  $\frac{\text{ash}}{\text{citric acid}}$  in the experiments here reported was about doubled.

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## The Utilization of Calcium Soaps in Rats.

By

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Several earlier authors have studied the influence of fat and fatty acids on calcium absorption. They have been compiled by BOYD, CRUM and LYMAN (1932). These authors studied the absorption of calcium soaps in young rats on a fat free diet and found on a calcium intake of 37 to 56 mg per rat per day the following utilization values for the various soaps: Calcium stearate 25 per cent, calcium palmitate 38 per cent, calcium oleate 90 per cent. On a calcium intake of 20 to 32.5 mg of calcium daily the utilization values were: Calcium stearate 45 per cent, calcium palmitate 65 per cent and calcium oleate 91 per cent.

WESTERLUND (1935) claimed that margarine as compared with butter has a deleterious effect on the absorption of calcium in rats. WIDMARK and NEYMARK (1936) supposed that the saturated fatty acids in margarine act unfavorably on the absorption of calcium. v. EULER et al. (1938, 1939) repeated the experiments of WESTERLUND in rats and found, that when the difference in vitamin D content between butter and margarine had been eliminated, there was no difference in their influence on the absorption of calcium. NICOLAYSEN (1938) reached the same conclusion in rats. He found no difference in the effect of margarine as compared with butter on calcium utilization in man, and that in rats hardened coco- and whalefat did not reduce the absorption of calcium.

Thus it appears that different types of fats exert little effect on the calcium absorption. Still the enormous differences found by BOYD et al. in the utilization of calcium in different soaps

remained unexplained and a reinvestigation was necessary. The degree of body saturation with calcium (NICOLAYSEN 1943) had to be taken into account.

### Experimental.

The following calcium soaps were used:

	Ca analysed	Ca calculated
Calcium palmitate . . . . .	6.6 %	7.3 %
» butyrate . . . . .	16.7 »	18.7 »
» oleate . . . . .	9.9 »	7.4 »
» stearate . . . . .	5.8 »	6.6 »
» lactate . . . . .	12.6 »	18.4 »

Three series of experiments were performed.

#### 1. The utilization of Calcium Soaps in Rats Unsaturated with Calcium but Given Ample Amounts of Vitamin D.

A group of 20 rats 8 weeks old with severe rickets (the rachitogenic diet see NICOLAYSEN 1943), were given the following diet for 1 month: 3 % egg albumin, 3 % brewer's yeast, 0.2 %  $\text{CaCO}_3$ , 0.2 % ferric citrate, 1 % NaCl, 92.5 % whole wheat flour. Each rat was given 2 g. meat and 20 I. U. vitamin  $\text{D}_2$  daily. The rats now weighed 160—220 g. They were next given the calcium "free" basal diet (NICOLAYSEN 1943). The absorption of 30 mg calcium as stearate was then compared with the absorption of 30 mg Ca as lactate in 1 week's experiment (technique: NICOLAYSEN and JANSSEN 1939). To each rat was weighed off 10 g diet + the calculated amount of stearate (according to previous analyses) daily. The diet was eaten quantitatively.

Table I gives the results.

Table I.  
*Mg Ca absorbed daily.*

Lactate	Stearate
30 . . . . .	24
30 . . . . .	26
30 . . . . .	26
32 . . . . .	27
32 . . . . .	27
32 . . . . .	27
32 . . . . .	28
32 . . . . .	28
32 . . . . .	28
33 . . . . .	30

Average: 31.5

27.1

## 2. The Utilization of Calcium Soaps in Rats Saturated with Calcium and Given Ample Amounts of Vitamin D.

A group of 30 rats were fed on a Ca and P rich diet with 20 I. U. vitamin D daily to each rat until they were 3 months old. Next the absorption of different soaps was studied as appears from Table II. Each of the five different soaps was first tested in 6 rats in one week's experiment. Then in another week the same soaps were tested in other groups of rats.

Table II.

*Mg Ca absorbed daily.*

Palmitate	Butyrate	Oleate	Stearate	Lactate
3	3	3	5	7
6	7	9	6	9
7	9	10	6	10
8	9	11	6	11
8	10	11	7	12
8	11	12	8	12
9	12	12	9	13
12	12	12	10	13
13	13	15	11	13
14	13	15	11	14
14	14	15	12	14
14	15	16	15	15
<hr/>				
Average: 9.7	10.7	11.8	8.8	11.9

## 3. The Utilization of Calcium Soaps in Vitamin D Deficient Rats.

A group of 30 rats, 6 weeks old with severe rickets were for two weeks given the Ca and P rich diet (NICOLAYSEN 1943) without any addition of vitamin D. After 3 days on the Ca "free" basal diet the absorption of calcium soaps was studied.

Table III gives the results.

Table III.

*Mg Ca absorbed daily.*

Stearate	Lactate	Oleate
13		
13		
15	14	13
15	15	15
15	16	15
15	16	15
16	18	16
17	18	17
18	18	17
19	18	18
19	21	20
<hr/>		
Average: 16	17	16



### Discussion.

It appears from table I that when rats are unsaturated with calcium and the absorption of calcium is very efficient, a statistically significant, but comparatively small difference is observed in the utilization of calcium stearate as compared with lactate. In rats saturated with calcium and consequently with a reduced ability to absorb calcium, the difference is reduced and only on the borderline of being statistically significant. In the lactate versus stearate experiments in Table II,  $t = \frac{11.9 - 8.8}{\sqrt{0.98^2 + 0.67^2}} = 2.55$ .

The other differences in these experiments were smaller and consequently without any significance.

The experiments give a clear picture of the significance of different degrees of body saturation with calcium as compared with the influence of different fatty acids on the absorption of calcium.

From a practical point of view it must be concluded, that the utilization of calcium soaps is largely independent of the nature of the fatty acid.

### Conclusion.

The utilization of calcium soaps as of other calcium salts is chiefly dependent on the body saturation with calcium and on vitamin D and largely independent of the nature of the fatty acids.

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## The Spectral Properties of the Visual Receptors of the Cat.

By

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The micro-electrode technique, used in several previous contributions to the problem of colour reception (GRANIT *et al.*, 1939—42), is particularly well suited for mammals on account of the ease with which large isolated spikes are obtained from the inside of their retinae. Rats (1941 a) and guinea pigs (1942 a) have previously been used in these experiments. Such spikes are here shown in fig. 1 for the cat's eye.

The cats have been decerebrated, given some 5—10 cc urethane (20 %), cornea and lens have been removed from one eye — the eye of the side on which the carotid artery has been left untied before decerebration — and the micro-electrode finally been inserted under the microscope in the usual manner. This combination of urethane and decerebration proved to be better than heavier anaesthesia or decerebration alone.

The large spikes, seen in fig. 1, are either well synchronized discharges from the axones of several ganglion cells or else impulses in single fibres. The all-or-none law is no reliable criterion for the degree of isolation as with the micro-electrode several factors, among them the influence of adjacent elements, less well placed relative to the electrodes, may cause changes in the size of the spikes. Differentiation between single and synchronized fibres — though theoretically important — cannot either be obtained by the analysis of the spectral properties of a given discharge of spikes because of the known existence of convergence of several

receptors towards the same ganglion cell. This cell may be the final common path for several rods as well as for rods and cones together (POLYAK, 1936). For this reason we have to expect both rod- and cone-properties from the same isolated spike, even if it belongs to a single fibre rather than to a number of well synchronized fibres. Actually all experiments up to date have proved this postulate to be correct.

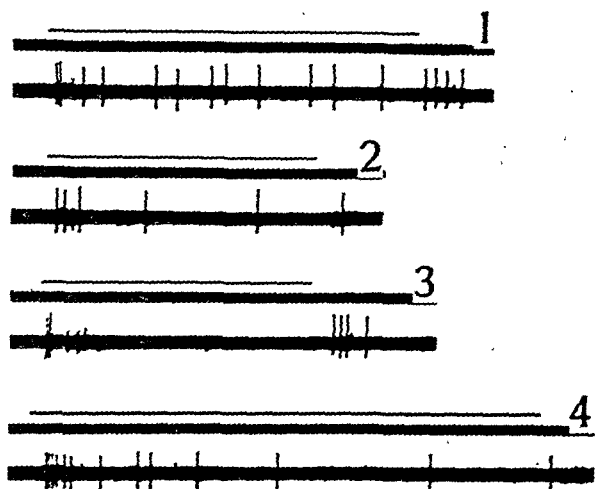


Fig. 1. In each record light signal, time in 50/sec. and spike activity.

- 1, stimulus of relative energy 6.3 at wave-length  $0.620 \mu$ ;
- 2, same at relative energy 2.3;
- 3, relative energy 2.5 at  $0.460 \mu$ .
- 4, from another experiment to illustrate element reacting merely to onset of illumination; wave-length  $0.500 \mu$ .

Fig. 1 refers to a particularly interesting, though not a very common type of response, in which an originally light-adapted eye had become dark-adapted during the experiment. The spikes illustrated had first given the distribution of sensitivity characterizing the so-called photopic dominator of fig. 3 whereupon, during dark-adaption, the sensitivity had risen greatly in the short wave-lengths. At this moment the records were taken. 1 and 2 are responses to stimulation with red light at relative energies 2.3 and 6.3, record 3 to stimulation with blue light at relative energy 2.5. These records are selected from a number of pictures in which a much larger range of relative energies from the threshold upwards led to the same characteristic difference in the responses to red and blue stimuli. Thus this difference could not be an intensity effect. The blue stimuli gave discharges only at "on" and "off" and nothing *during* illumination, the

red stimuli elicited a train of impulses *during* illumination in addition to increasing the frequency at "on" and "off". No difference could be seen between the spikes set up in the ganglions by the differently coloured stimuli and yet the red end of the spectrum must have activated the discharging fibre through another channel than the blue one.

Alternatively, as stated, the spikes, though they look as if coming from a single fibre, may have come from well synchronized elements, split up into separate fibres by the differently coloured stimuli. But in the latter case one would expect the spikes to decrease in size, when light from the ends of the spectrum are used as stimuli, because the rod and cone ranges only overlap in the middle of the spectrum. Visual purple, for instance, has a negligible effect from  $0.620 \mu$  further out towards the red. But even though the size of the spikes somewhat varies, it does not vary with wave-length. True convergence of rods and cones towards the same ganglion cell is therefore the probable explanation of the experiment illustrated in fig. 1.

True convergence is also the most probable explanation of the fact that in the cat as well as in all other animals with mixed eyes the absorption curve for visual purple sooner or later will determine the distribution of sensitivity of almost any discharge of spikes after some time in the dark. With the light-adapted cat this is a particularly serious source of error on account of the rapidity with which dark-adaptation often takes place, and steps have to be taken to counteract the influence of dark-adaptation. The presence of the broad dominator band of sensitivity in the cat's eye in light-adaptation (see fig. 3) makes checking of dark-adaptation more important than in the mammals hitherto studied though in principle the procedure is the one already used in several previous contributions to this problem.

### Procedure.

A large spike was located. With the electrode inserted the animal was then left in the illumination from the microscope-lamp, placed above the eye (2,400 m.c.), for 10 min., sometimes longer. It was then allowed to recover, and its eye was illuminated by brief flashes of light of different wave-lengths at intervals of 10 sec. in order to measure the energy necessary for the threshold in each wave-length. Recovery curves during dark-adaptation were plotted from these data in terms of energy reciprocals against time in the dark for a number of regularly recurring wave-lengths (cf. the eye of the rat, GRANIT, 1941 a). Between these curves stray observations on other wave-lengths were inserted

into the diagrams at their appropriate time moments. From these diagrams the distribution of sensitivity could be calculated.

A characteristic of such diagrams, of which some have been published in the work on the rat's eye (1941 a), is that sooner or later the sensitivity in the short wave-lengths, best represented by wave-length  $0.500\ \mu$ , which is at the top of the absorption curve for visual purple, begins to increase at a very much faster rate than the sensitivity to light of long wave-lengths. Different réceptors register this change after different intervals in the dark. Generally there is a clear break in the curve, as shown already with the rat's eye (GRANIT, 1941 a) and also with the frog's eye (GRANIT, 1941 b), after which the rate of rise of sensitivity becomes particularly fast for wave-lengths around  $0.500\ \mu$ . The turning point in the cat's eye often comes after about 10—15 min. in the dark. Sometimes the break is less well marked, the rate of rise of sensitivity being fast from the beginning, sometimes the change in relative sensitivity to long and short wave-lengths is very much delayed. When this is so a relatively pure cone population may have been struck or else deficient circulation, caused by the decerebration, may have retarded the regeneration of visual purple (ZEWI, 1939).

If the experiments are to reproduce the true photopic distribution of sensitivity, the result must either be obtained within the first 10—15 min. or else controls must have demonstrated that the experiment refers to a type of element with delayed dark-adaptation proper, defined as delayed rise of sensitivity for wave-length  $0.500\ \mu$ .

Applying these principles it is possible to obtain from each diagram a series of values illustrating the photopic distribution of sensitivity. This is the definition of the term *series* used in all this work. Sometimes the same spike could be kept under the electrode for repeated re-adaptation to light. This gave one or several new sets of values thereby increasing the accuracy with which a series could be determined. Behind a series can thus be a greater or smaller number of readings of which each series is a final average. Several series of similar distribution of sensitivity have generally been averaged in the presentation of the results.

### The Photopic and Scotopic Spectra.

LYTHGÖE's (1937) absorption curve for visual purple is drawn in fig. 2. The circles around it represent 31 averaged values, based on 102 readings from 4 series in 4 dark-adapted cats. The scotopic values are relatively too high in  $0.540$ — $0.580\ \mu$ . The maximum of this increase around  $0.560\ \mu$  is perhaps due to influence from the photopic dominator, shown in fig. 3. But back reflexion from the *tapetum lucidum* provides another probable explanation of this hump in the scotopic curve.

The most striking fact about the light-adapted eye of the cat is that it possesses the broad photopic dominator band of sensitivity with maximum around  $0.560 \mu$  (fig. 3), previously seen in the eyes of frog (GRANIT, 1941 b), snake (GRANIT, 1942 c) and pigeon (GRANIT, 1942 b), but not in the rat (GRANIT, 1941 a) and the guinea pig (GRANIT, 1942 a). These two are the only mammals hitherto studied. The cat is thus the first mammal with photopic vision well enough developed to be represented by this very characteristic dominator band. The finding is important since it demonstrates that the principles discovered for lower vertebrates also are valid for mammals.

The large circles of fig. 3 and the curve drawn between them refer to 4 particularly good series, good in the sense that the dominator was little if at all influenced by incipient dark-adaptation and that the values in the short wave-lengths were recorded at a very early stage of the recovery in the dark after light-adaptation.

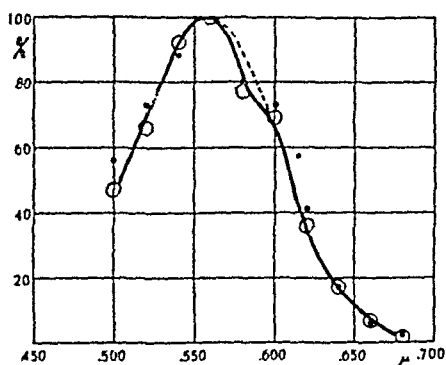


Fig. 3. Photopic dominator of cat, as described in text.

The great majority of them represented single spikes of the kind shown in fig. 1.

There is a regularly recurring asymmetry in the dominator which in fig. 3 has been filled out by a broken line. Completed

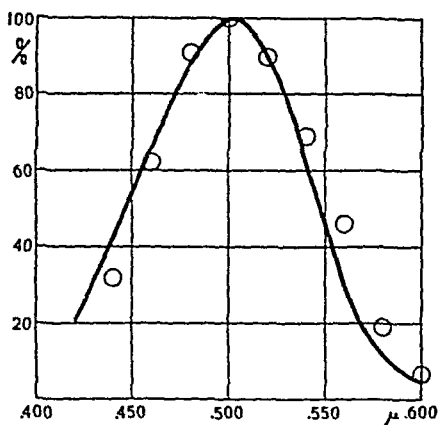


Fig. 2. LYTNGOE's (1937) absorption curve for visual purple. Circles from experiments on 4 scotopic cats (see text).

The black dots refer to 87 averages collected from 188 readings representing all the twelve series in which the dominator with maximum in  $0.560 \mu$  was reasonably free from influence from the absorption curve for visual purple. A slight rise in the green is just noticeable. As there were 33 photopic series the dominator has been present in 36 % of the photopic elements analyzed.

in this manner the curve is identical with the dominator found in the frog's eye. What is the reason for the asymmetry?

In order to answer this question we must first consider the organisation of colour reception as revealed by these experiments on different animals. Perfect colour reception seems to necessitate a dominator band for the perception of luminosity and a number of narrow modulator bands in different regions of the spectrum to modulate the impression of luminosity (white) to colour (GRANIT, 1941 c). One of the most regularly recurring modulators, seen in rats, frogs and snakes, has its maximum in  $0.600 \mu$ . The asymmetry in the dominator curve of fig. 3 could be due to unsuccessful isolation or deficient development of this modulator which therefore only would be capable of pushing up a hump in  $0.600 \mu$ . Up till now it has always been possible to isolate this modulator in those animals where it has been present. But the narrow "red" modulator has not been found in the isolated state in the cat's eye. The hump in this region therefore suggests some defect in the development of the modulator. The dominator may also be somewhat modified by back reflexion from the *tapetum* leading to a relative increase of stimulus strength in the region of the spectrum that is reflected.

If the *tapetum* be removed from the eye for examination it is found to reflect a dominantly yellow-green light. To my eye pure yellow is in  $0.580 \mu$ . Yellow-green would therefore correspond fairly well to the maximum of the dominator in  $0.560 \mu$  which thus by back reflexion may have received an additional stimulus making it possible to diminish the energy used for the spectral stimulus in this region. This in turn would lead to an accentuated top of the dominator curve. The *tapetum* of the cat's eye is a very efficient reflector, especially by comparison with the black pigment which in most eyes absorbs stray light of all wave-lengths. It is therefore reasonable to expect it to modify the curves in the region where it reflects maximally. The rise in the scotopic curve of fig. 2 in the same region suggests that in both cases the same factor is at work. Therefore a common explanation seems probable.

Some of the experiments for which the early values after light-adaptation were plotted in fig. 3 were continued, despite incipient dark-adaptation, for some time afterwards in order to illustrate the expansion of the dominator towards the short wave-lengths. In fig. 4 the filled circles illustrate 63 averages from 7 series of

this type (119 readings). The curve is drawn *between* the values for  $0.520\ \mu$  and  $0.540\ \mu$  but the indication of a hump in  $0.520\ \mu$  is probably significant. The asymmetry to the right of  $0.560\ \mu$  is still noticeable.

So far we have only been concerned with 36 % of the photopic series, those possessing their maximum in  $0.560\ \mu$ . In the remaining 64 % light-adaptation did not succeed in pushing the maximum further to the right than to  $0.520\ \mu$ . These curves have always had a secondary maximum in  $0.560\ \mu$ . As such curves were obtained in the majority of our experiments the maximum for all 33 photopic series averaged together lies in  $0.520\ \mu$ . The average photopic curve obtained in this manner is shown in fig. 4 (line between open circles).

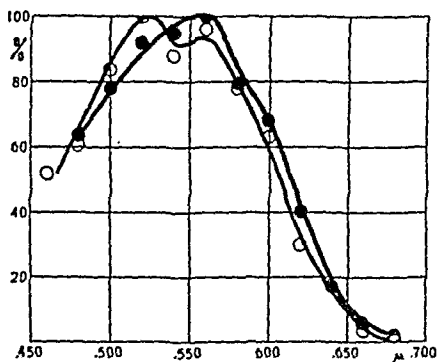


Fig. 4. Averages, as described in text.

In this curve 425 readings from 13 cats have been collected into 200 averages which in their turn have been averaged for the values plotted (open circles). The result means that the dominators have been too few to effect a complete Purkinje shift but sufficiently many to create a relatively large secondary hump in  $0.560\ \mu$  and give the eye a considerable sensitivity to red light.

As a matter of fact PIPER (1905) failed to find a Purkinje shift with the electroretinogram as index but the reason for this may also have been that he did not realize what precautions have to be taken to ensure light-adaptation in an animal of this type. To the absence of a Purkinje shift in PIPER's experiments may also have contributed the fact that the electroretinogram to a stimulus spreading diffusely over the retina is an average from the whole retina including the periphery where cones may be scarce or absent. Finally, in the author's experience (GRANIT, 1935, GRANIT, MUNSTERHJELM and ZEWEI, 1939) really efficient light-adaptation in cats almost removes the b-wave or at any rate makes it so small that within the first 10 min. after light-adaptation quantitative work with the electroretinogram as index is very difficult.

With a method based on animal behaviour MURR (1932) succeeded in demonstrating *after light-adaptation* a shift of the luminosity curve of the order of  $0.015\ \mu$  towards the long wave-lengths but his results for the completely *dark-adapted* eye, when corrected for equal quantum intensity and compared with visual purple absorption, are difficult to



understand without assuming experimental errors of some kind. It is an exceedingly narrow curve with maximum around  $0.525 \mu$ , and in  $0.480 \mu$  at only about 30 % as compared with 88 % for visual purple. MURR only knew the energy of his stimuli at the light source and relied upon it being similarly distributed after passage through the spectro-scope and reflexion from the grey paper covering the food which the animals had to locate. This fact must be responsible for considerable errors in the calculation of the energy of the spectral light reflected to the eye.

The average photopic curve of fig. 4 contains the dominator and at least one additional sensitivity curve combined with it. It is possible to show that this second curve in the green cannot be an unmodified curve for visual purple absorption. This can be done by adding to the dominator the absorption curve for visual purple in different proportions and plotting on a percentage basis the complex curves obtained. These do not show the sharp hump in  $0.520 \mu$ , seen in the average curve of fig. 4, though actually, with certain proportions between dominator and visual purple, the maximum is located in  $0.520 \mu$ .

From the experiments with rats and guinea pigs it is known that after light-adaptation a number of elements are found to be characterized by sensitivity curves which look like abnormally narrow curves for visual purple absorption. If similar elements are found in the cat's retina in sufficient numbers they would combine with the dominator to give just the kind of curve shown in fig. 4.

The curves of fig. 5, both referring to early values for single spikes in the light-adapted eye show that this explanation of the

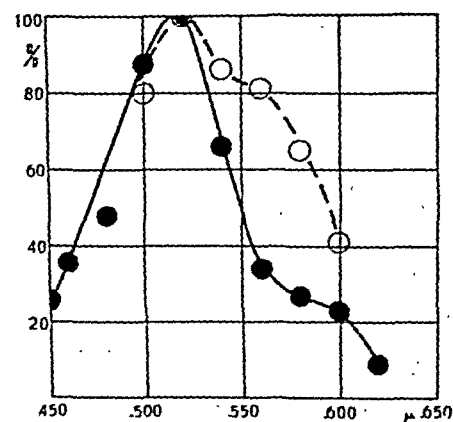


Fig. 5. Two experiments on single spikes showing combination of narrow "green" curve with dominator. See text.

hump in  $0.520 \mu$  probably is correct. Influence of the dominator is very marked in the curve drawn in broken lines, less marked in the curve drawn in full which is the narrowest one ever obtained in the experiments with the cat. Both show that a narrow band of modulator type can be superimposed upon the dominator. The maximum of this modulator is between  $0.500$  and  $0.530 \mu$  in these curves. But con-

sidering that on the right side of the maximum the dominator contributes to the total result, it is necessary to place the maximum of the modulator nearer to  $0.500 \mu$ . This means that it must be identical with the modulator seen previously in guinea pigs and rats and there described as an abnormally narrow absorption curve for visual purple.

Thus, in order to explain all our results with the light-adapted retina of the cat, it has only been necessary to supplement the demonstration of the existence of a dominator and a green modulator by the assumption that they combine so that the average photopic curve of fig. 4 is dominated by an abnormally narrow absorption curve for visual purple upon which is superimposed the dominator, slightly modified by back reflexion from the *tapetum lucidum*. On account of the dominator the maximum of the average curve is shifted to  $0.520 \mu$  instead of being in  $0.500 \mu$  where it is found in guinea pigs and rats which are lacking the dominator.

### Colour Reception.

SCHULTZE (1866) stated that the cat's retina contains 2 or 3 times more rods per cone than the human eye. Considering that in the eye of the guinea pig, which according to the same author is lacking cones, the dominator band and all red-sensitive elements are absent, and that, further, the dominator, where it is found, is responsible for the Purkinje shift in light-adaptation, it is clear that the dominator must be located to the cones. The narrow "green" band with the maximum of visual purple absorption has been seen in the guinea pig (GRANIT, 1942 a) and in the rat (GRANIT, 1941 a). It must therefore be located to the rods and probably is due to a slight modification of the visual purple molecule. That this band can be used by different animals in the state of light-adaptation seems clear. But whether it actually serves as a modulator for green, in every respect comparable with the "red" modulator in  $0.600 \mu$  and the "green" one in  $0.530 \mu$ , is perhaps less certain. If it does, the cat may be able to discriminate colours in the green region of the spectrum, though this discriminative mechanism must be primitive. The lack of a definite "red" modulator suggests relatively incomplete discrimination also in the red. However the experiments have not definitely excluded red-sensitive modulators. The hump in  $0.600 \mu$  suggests inadequate development of such elements.

The presence of the dominator in the animals, previously studied, has indicated good colour vision. By this criterion the cat's retina is an improvement upon the retinae of guinea pigs and rats. If the modulators are few they may be difficult to discover, and, yet, the animal be able to discriminate colours with sufficiently strong stimuli. But no doubt even the presence of the dominator *without* modulators must be regarded as an advantage as through this band a large range of the spectrum becomes available in daylight at a level of intensity diminishing the usefulness of visual purple.

Most mammals with cones have some kind of *area centralis* in which cones are present in larger quantities than elsewhere. The relative number of rods and cones in the area which the cat uses in day-light will determine whether the maximum of its photopic luminosity curve is around  $0.560 \mu$  or around  $0.520 \mu$  or occupies some place between these two points. Considered from the point of view of the human eye the cat would thus, if colour blind, either be a *deuteranope* or a *protanope*, both states being defined as red-green blindness, the former with the luminosity curve of normal subjects, the latter with the luminosity curve shifted towards the short wave-lengths. It is also possible that the cat, in case it possessed some red modulators, is better defined as merely being derteranomalous or protanomalous. These states would be slightly modified by *tapetum* reflexion.

Cats are generally regarded to be colour-blind (STAGNER, 1931). So is the human periphery. But if strong lights are used the human periphery becomes colour-sensitive suggesting that it merely is a question of the relative number of modulators in different regions of the retina. Similarly the cat, with a very limited number of modulators, may be sensitive to colour if it is well light-adapted and very strong stimuli are used. In testing this properly atropine would have to be used to keep the pupil well opened as otherwise the contraction of it to a small slit may prevent both light-adaptation and sufficiently strong stimulation.

### Summary.

The micro-electrode technique has been used for the recording of spikes of activity from the retina of the cat in order to study the distribution of sensitivity to spectral light of the retinal elements.

In dark-adapted cats the distribution of sensitivity corresponds to the absorption curve for visual purple.

In light-adapted cats some elements give the broad so-called photopic dominator band with maximum in  $0.560\ \mu$ , others a narrow curve with maximum in  $0.520\ \mu$  combined with the dominator in different ratios.

The narrow curve is probably identical with the band seen in the eyes of rats and guinea pigs corresponding to an abnormally narrow absorption curve for visual purple, in the cat shifted somewhat to the right because of the added effect of the dominator.

Some results indicate that back reflexion from the *tapetum lucidum* plays a rôle in determining the shape of the curves in the yellow-green region of the spectrum.

The colour vision of the cat has been discussed in the light of the results arrived at.

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# Die Wirkung von Pervitin auf den Kreislauf und die Atmung beim Menschen.

Von

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Während der letzten Jahre haben sich verschiedene Derivate aus der Adrenalin-Ephedringruppe grosses Interesse zugezogen. Durch konsequente Molekularstrukturveränderungen hat man eine Serie Präparate hergestellt, deren Eigenschaften man in gewisser Hinsicht schon im voraus angeben konnte. Für alle diese gemeinsam ist ein Phenyl- und ein Alkylaminradikal. Nach BARGER und DALE (1910) nimmt die sympathicomimetische Wirkung mit der Anzahl der Hydroxylgruppen zu, besonders derjenigen, die an den Ring gebunden sind. Dagegen nimmt die Haltbarkeit und die Wirkungsdauer ab. Das wenig haltbare Adrenalin hat eine sehr kräftige Wirkung auf den Kreislauf, während Ephedrin nicht so stark kreislaufwirksam, dagegen besser haltbar und per os wirksam ist. Sympatol und Veritol, die eine Zwischenstellung einnehmen, wirken vor allem auf den Kreislauf und sind ausserdem per os wirksam. Benzedrin und Pervitin, die keine Hydroxylgruppen haben, und ziemlich schwer zerstörbar sind, wirken stark erregend auf das zentrale Nervensystem, dabei sind sie noch ziemlich stark kreislaufwirksam.

In früheren Arbeiten in dem hiesigen Laboratorium wurde eine Steigerung des Blutstroms durch Adrenalin, Ephedrin, Sympatol (v. EULER und LILJESTRAND, 1927 und 1929) sowie durch verschiedene Oxyephedrine (LILJESTRAND und LINDE, 1933) nachgewiesen. Die Wirkung des Benzedrins auf das Herzminutenvolumen wurde von BERGGREN und SÖDERBERG (1938) unter-

sucht. Sie fanden nach einer Dosis von 20 mg. per os eine mässige Steigerung des Sauerstoffverbrauchs (etwa 10 %), eine Herabsetzung der arteriovenösen Sauerstoffdifferenz und eine Steigerung des Minutenvolumens. Der systolische Blutdruck wurde etwa 25% erhöht, der diastolische nur wenig.

Pervitin, das sich vom Benzedrin durch eine Methylgruppe an der Aminogruppe unterscheidet, soll nach HAUSCHILD (1938) noch stärker erregend auf das zentrale Nervensystem wirken. Es ist aber auch kreislaufwirksam. Im Tierversuch fand HAUSCHILD eine Blutdrucksteigerung, die mindestens ebenso stark war wie die des Ephedrins, jedoch von längerer Dauer. Beim Menschen hat man nach Dosen von 9—15 mg. per os eine mehrere Stunden anhaltende Blutdrucksteigerung gefunden (SCHOEN 1938, PÜLLEN 1939, SEIFERT 1939). SEIFERT fand nach 15 mg. auch eine Steigerung der Ventilation von 8.6 bis zu 12.9 l/min. bzw. von 5.6 bis zu 12.9 l/min., und eine Steigerung des respiratorischen Quotienten bis über 1, die mehrere Stunden anhielt. Diese Wirkung ist nicht von anderer Seite bestätigt worden. LEHMANN, STRAUB und SZAKALL (1939) beobachteten in Ruheversuchen keine Änderung des Stoffwechsels, der Atmung und des Kreislaufs. Im Arbeitsversuch fanden sie eine geringe Neigung zur Hyperventilation. In einer Reihe von Versuchen bestimmten sie auch entsprechend dem Vorgehen von Knipping Schlag- und Minutenvolumen des Herzens, sahen aber keinerlei Einfluss von Pervitin. Im übrigen scheint es keine Untersuchungen über den Einfluss des Pervitins auf das Herzminutenvolumen zu geben.

## Eigene Untersuchungen.

### Methodisches.

Als Versuchspersonen dienten 7 gesunde Studenten im Alter von 18—27 Jahren. Es wurden 11 Versuche ausgeführt. Pervitin wurde in intramuskulärer Injektion in einer Dosis von 0.21 mg. pr Kg. Körpergewicht gegeben, entsprechend 15 mg. bei 70 Kg. Um einheitlichere Versuchsbedingungen zu bekommen, wurde die parenterale Applikation gewählt. Die Versuche wurden unter Standardbedingungen ausgeführt. Zuerst wurden zwei Normalbestimmungen gemacht (in den Tabellen mit I und II bezeichnet). Dann wurde Pervitin injiziert und nach 20—30 Min., 50—60 Min. und 80—90 Min. neue Bestimmungen ausgeführt (III, IV und V). Bei jeder Bestimmung wurde erst der Radialis-puls gezählt und der Blutdruck mittels Methylenjodidmanometer nach <sup>7</sup>ALJESTRAND und ZANDER (1928) festgestellt. Ventilation, Sauerstoff-

Tabelle

	Vor der Inj. (I und II)					20—30 Min. nach der Inj. (III)				
	O <sub>2</sub> - Verbr. ml/Min.	Art.- ven. O <sub>2</sub> -Diff. ml/lit	Min.- Vol. lit/Min.	Venti- lation lit/Min.	R. Q.	O <sub>2</sub> - Verbr. ml/Min.	Art.- ven. O <sub>2</sub> -Diff. ml/lit	Min.- Vol. lit/Min.	Venti- lation lit/Min.	R. Q.
W. D.	224	51	4.4	6.90	0.79					
♂ 23 J.	242	51	4.8	7.64	0.79	225	44	5.1	7.20	0.82
G. J.	202	63	3.2	6.71	0.79	317	60	5.3	18.88	1.42
♂ 23 J.	213	68	3.1	6.98	0.78					
"	209	68	3.1	7.43	0.83	253	47	5.4	13.12	1.13
	202	61	3.3	7.01	0.80					
A. F.	196	52	3.8	6.60	0.81	212	41	5.2	6.20	0.75
♀ 21 J.	205	49	4.1	5.83	0.76					
G. F.	251	52	4.8	6.60	0.77	292	53	5.5	7.90	0.76
♂ 18 J.	258	51	5.0	6.66	0.80					
M. B.	210	62	3.4	5.29	0.68	256	54	4.8	7.09	0.66
♀ 27 J.	212	64	3.3	5.26	0.65					
A. D.	249	62	4.0	5.83	0.75	248	54	4.7	7.14	0.89
♂ 24 J.	239	63	3.7	6.33	0.76					
"	219	77	2.9	6.01	0.80	262	57	4.6	7.62	0.84
	221	80	2.8	6.05	0.82					
N. S.	257	57	4.5	8.05	0.89	302	49	6.2	12.62	1.12
♂ 22 J.	270	—	—	8.58	0.89					
"	264	54	4.9	7.78	0.77	324	39	8.2	14.39	0.99
	—	—	—	—	—					
"	259	68	3.8	7.19	0.81	281	45	6.3	6.10	0.76
	277	50	5.6	7.51	0.78					
Mittel:	234.0	59.7	4.01	6.823	0.785	270.2	49.1	5.59	9.85	0.921

verbrauch und respiratorischer Quotient wurden durch Sammlung der Expirationsluft in einem grossen Spirometer und nachfolgender Gasanalyse ermittelt. Schliesslich wurde die arteriovenöse Sauerstoffdifferenz nach GROLLMANS (1932) Acetylenmethode bestimmt.

### Experimentelles.

Die psychisch erregende, euphorisierende Wirkung, wie sie von zahlreichen anderen Autoren geschildert ist, setzte etwa 10—15 Min. nach der Injektion ein und war am Ende des Versuches unvermindert. Bei der Versuchsperson M. B. war 15—45 Min. nach der Inj. eine starke psychische Unruhe mit Angst und Depression vorhanden, auf die dann der gewöhnliche psychische Effekt folgte.

In der Tabelle 1 werden einige ursprüngliche Versuchsergebnisse angegeben, in Tab. 2 die Mittelwerte und die Differenzen für

1.

50—60 Min. nach der Inj. (IV)					80—90 Min. nach der Inj. (V)				
O <sub>2</sub> - Verbr. ml/Min.	Art.- ven. O <sub>2</sub> -Diff. ml/lit	Min.- Vol. lit/Min.	Venti- lation. lit/Min.	R. Q.	O <sub>2</sub> - Verbr. ml/Min.	Art.- ven. O <sub>2</sub> -Diff. ml/lit.	Min.- Vol. lit/Min.	Venti- lation lit/Min.	R. Q.
242	44	5.5	7.59	0.82	255	42	6.0	7.60	0.72
249	57	4.4	13.37	1.18	244	43	5.7	13.03	1.24
224	57	3.9	8.95	0.91	239	60	4.0	10.92	0.98
208	43	4.9	5.19	0.69	209	41	5.2	5.65	0.73
267	40	6.7	6.90	0.68	275	53	5.2	7.60	0.83
282	70	4.0	8.51	0.72	245	64	3.8	6.93	0.68
231	68	3.4	6.30	0.81	234	59	4.0	6.20	0.78
242	56	4.3	7.00	0.81	239	71	3.4	6.86	0.81
275	63	4.4	8.45	0.83	281	60	4.7	9.54	0.91
278	44	6.4	9.03	0.79	281	59	4.8	8.16	0.76
290	50	5.8	6.94	0.74	290	48	6.1	7.48	0.73
253.5	53.7	4.88	8.021	0.808	253.8	54.3	4.81	8.179	0.833

sämtliche untersuchten Funktionen. Die beiden Normalbestimmungen zeigen keinen statistischen Unterschied (9 Versuche), mit Ausnahme vom Sauerstoffverbrauch, wo man eine kleine statistisch wahrscheinliche Zunahme von I bis II findet, 6.3 ml + 3.03 (2.7 %), die man wohl übersehen kann.

Bei der Beurteilung des Effekts wurde in jedem Versuche die Differenz zwischen den Werten nach der Injektion und dem Mittelwerte der beiden Normalbestimmungen, sowie der Mittelfehler dieser Differenzen, berechnet. Auf diese Weise benutzt man den Umstand, dass die Normalbestimmungen und die Bestimmungen nach der Injektion bei denselben Personen und bei der gleichen Gelegenheit ausgeführt wurden.

In der Literatur gibt es, wie bereits oben gesagt, verschiedene Ansichten über die *Einwirkung auf die Ventilation* und den respiratorischen Quotienten. Wie aus der Tab. I hervorgeht, reagierten zwei der Versuchspersonen, G. J. und N. S., mit einer bedeutenden



Tabelle 2.

Funktion	Vor der Inj. I + II 2	1/2 Stunde nach der Inj. (III)	Diff. I + II III - 2	1 Stunde nach der Inj. (IV)	Diff. I + II IV - 2	1 1/2 Stunde nach der Inj. (V)	Diff. I + II V - 2
O <sub>2</sub> -Verbr. . .	234.0	270.2	+36.3 ±9.66 +15.5%	253.5	+19.5 ±6.47 +8.3%	253.8	+19.6 ±3.92 +8.4%
ml/Min. . .							
Veränderung							
Art.-Ven.							
O <sub>2</sub> -Diff. . .	59.7	49.1	-10.5 ±1.86 -17.6%	53.7	-6.0 ±2.68 -10.0%	54.3	-5.3 ±2.43 -8.9%
ml/Lit. . .							
Veränderung							
Min.-vol. . .	4.01	5.59	+1.58 ±0.243 +39.4%	4.88	+0.88 ±0.206 +22.0%	4.81	+0.80 ±0.210 +19.9%
Lit./Min. . .							
Veränderung							
Schlagvol. . .	75.5	94.7	+19.3 ±5.32 +25.6%	83.7	+8.2 ±3.99 +10.9%	79.3	+3.8 ±4.41
ml. . . . .							
Veränderung							
Pulsfrequenz .	54.1	60.6	+6.5 ±1.47 +12.0%	60.0	+5.9 ±1.99 +10.9%	63.0	+9.0 ±2.95 +16.6%
Veränderung							
Ventilation .	6.823	9.850	+3.027 ±1.1715 +44.4%	8.021	+1.198 ±0.6310 +17.5%	8.179	+1.356 ±0.5884 +19.9%
Lit./Min. . .							
Veränderung							
Resp. Quot. .	0.785	0.921	+0.136 ±0.0615 +17.3%	0.808	+0.023 ±0.0418	0.833	+0.047 ±0.0449
Veränderung							
Syst. Bldr. .	113.2	133.9	+20.7 ±2.39 +18.2%	136.8	+23.6 ±4.13 +20.8%	131.2	+18.0 ±4.26 +15.9%
mm. Hg. . .							
Veränderung							
Diast. Bldr. .	80.5	95.5	+15.0 ±3.32 +18.6%	95.6	+15.2 ±3.48 +18.9%	89.3	+8.8 ±3.26 +10.9%
mm. Hg. . .							
Veränderung							
Mittelbldr. .	96.6	114.9	+18.3 ±2.66 +18.9%	116.4	+19.7 ±3.83 +20.4%	110.6	+13.9 ±3.27 +14.4%
mm. Hg. . .							
Veränderung							

Zunahme der Ventilation und des Quotienten, bei G. J. in allen drei Bestimmungen nach der Injektion, bei N. S. hauptsächlich 1/2 Stunde nach der Injektion. Dieselben Ergebnisse wurden in der Hauptsache bei Wiederholung der Versuche gefunden. Die Hyperventilation in diesen Fällen war schon nach 10—15 Min. merkbar, gleichzeitig wie die Blutdruckwirkung angefangen hatte.

Die Resultate werden aus der Tab. 2 ersehen. Der Sauerstoffverbrauch wurde mässig erhöht, maximal 1/2 Stunde nach der Injektion mit etwa 15 % der Normalwerte. Die Zunahmen sind statistisch sichergestellt. Die arteriovenöse Sauerstoffdifferenz sank, am stärksten nach 1/2 Stunde, etwa 18 %. Die späteren Abnahmen waren geringer und nur statistisch wahrscheinlich. Das Herzminutenvolumen stieg nach 1/2 Stunde mit 39 %. Nach

1 und  $1\frac{1}{2}$  Stunde war die Steigerung nur noch etwa 20 %. Die Änderungen sind statistisch sichergestellt. Das Schlagvolumen wurde nach  $\frac{1}{2}$  Stunde mit 26 % erhöht, nach 1 Stunde mit 11 %, das letzte nur statistisch wahrscheinlich.

Die Ventilation zeigte nach  $\frac{1}{2}$  Stunde eine statistisch wahrscheinliche Steigerung von 44 %, offenbar wegen der hohen Werte von G. J. und N. S. Wenn man von diesen absieht, erhält man bei den restlichen 6 Versuchen eine praktisch sichergestellte Zunahme von  $0.952 \text{ l/Min.} \pm 0.3191$ , was 13 % der Normalwerte entspricht. Der respiratorische Quotient zeigte eine statistisch wahrscheinliche Zunahme von 17 % nach  $\frac{1}{2}$  Stunde. Wenn man von G. J. und N. S. absieht, findet man keine nachweisbare Zunahme,  $+0.023 \pm 0.0241$ . Auch im übrigen veränderte sich nicht der Quotient. Im Durchschnitt scheint also Pervitin keine Einwirkung auf die Ventilation zu haben ausser einer möglichen leichten Steigerung, die etwa dem erhöhten Sauerstoffverbrauch entspricht. Wie man sieht, können doch einzelne Individuen mit einer kräftigen Steigerung der Ventilation reagieren.

Die Blutdruckszunahme war am stärksten nach 1 Stunde, etwa 20 %, dann sank sie. Der systolische, diastolische und Mittelblutdruck nahmen ungefähr im gleichen Masse zu. Die Steigerungen sind statistisch sichergestellt. Die Pulsfrequenz wurde mässig erhöht und war nach  $1\frac{1}{2}$  Stunde noch im Steigen, im Gegensatz zu sämtlichen anderen Funktionen.

### **Zusammenfassung.**

In 11 Versuchen an 7 gesunden Versuchspersonen wurde die Wirkung von Pervitin auf Kreislauf und Atmung untersucht. Pervitin wurde in intramuskulärer Injektion in einer Dosis von 15 mg. per 70 kg. Körpergewicht gegeben. Das Herzminutenvolumen wurde mittels der Acetylenmethode von GROLLMAN bestimmt.

Eine halbe Stunde nach der Injektion wurde eine Steigerung des Herzminutenvolumens von 39 % des Normalwertes festgestellt, die nach 1 und  $1\frac{1}{2}$  Stunde immer noch etwa 20 % ausmachte. Der Sauerstoffverbrauch stieg mässig. Die Ventilation und der respiratorische Quotient wurden in den meisten Fällen nicht beeinflusst, aber zwei der Versuchspersonen reagierten mit einer kräftigen Hyperventilation und einer Steigerung des Quotienten bis über 1.

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## Rate of Formation of Nucleic Acid in the Organs of the Rat.

By

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The rate of formation of nucleic acid of the thymus nucleic acid type was investigated in the organs of the rat by administering labelled phosphate to rats and by determining the labelled P content of the desoxyribose nucleic acid extracted from the organs after the lapse of some days. The percentage of labelled nucleic acid present indicates the percentage of the total nucleic acid of the organs which is built up in the course of the experiment, as described in this note.

Preliminary figures on the rate of formation of nucleic acid in some of the organs of the rabbit were communicated at an earlier date (HAHN and HEVESY 1940). Data are furthermore available on the rate of formation of labelled "nucleoprotein" in some organs of the mouse (TUTTLE, ERF and LAWRENCE 1941). In our previous work, we extracted the nucleic acid with sodium chloride solution. TUTTLE and his colleagues removed the acid soluble and the phosphatide P fractions from the organs investigated and considered the residual P to be phosphorus of the "nucleoprotein" fraction. Extended studies carried out in this laboratory lead to the result that it is hardly possible to obtain nucleoprotein sufficiently purified from non-nucleoprotein phosphorus by the last mentioned procedure. Muscles and other organs of the frog containing labelled P were treated for weeks daily alternately with trichloroacetic acid solution and with ether alcohol mixtures. The specific activity of the remaining "nucleo-

protein" P was determined subsequently. It was found much higher than the specific activity (activity per mg P) of phosphorus obtained from properly purified nucleic acid. As shown in this note, the rate of formation of nucleic acid in most organs is very slow and, correspondingly, the specific activity of the nucleic acid P few hours and even some days after the administration of labelled P is very low also.

After the lapse of 2 hours, 1 mg nucleic acid P of the liver of the rat, for example, contains but  $2 \cdot 10^{-4}$  per cent of the labelled phosphorus administered, while the corresponding figure for 1 mg acid soluble P of the liver is about 1 per cent. If only  $10^{-3}$  part of the isolated nucleic acid P is composed of acid soluble P present as an impurity, a grossly erroneous value will be found for the specific activity of the nucleic acid P, viz.  $12 \cdot 10^{-4}$  instead of  $2 \cdot 10^{-4}$ . This example illustrates the necessity of an exceedingly careful purification of the nucleic acid fraction from all non-nucleic acid phosphorus. In our work, we are not faced with the great difficulties which were surmounted by HAMMARSTEN in his experiments which lead to the preparation of nondepolymerized nucleic acid. On the other hand, we have to avoid the presence of even minimal amounts of non-nucleoprotein P, the presence of which in any other but the radioactive investigations would certainly not be found disturbing.

### Experimental Procedure.

We applied the method of extraction and purification described by KLEIN and BECK (1935) adapted to work with tissue containing radioactive phosphorus, as previously used by H. VON EULER and one of the present writers (1942) in their work on the rate of formation of nucleic acid in the Jensen sarcoma of the rat. The washed tissue is stirred with an equal weight of 5 per cent sodium chloride solution brought to boiling. Acetic acid is added until the major part of the proteins present is precipitated. Sodium acetate and sodium hydroxide are then added and the alkaline solution is heated until the tissue is dissolved.

The next operation is carried out in a slightly acid solution. This is obtained by adding acetic acid. From this solution, the protein present is removed by adding a dialysed colloidal iron hydroxide solution containing 5 per cent  $\text{Fe}_2\text{O}_3$ . An excess of acetic acid is added and the hot solution is filtered. By adding an equal volume of methylalcohol to the filtrate, the crude nucleic acid precipitates.

The crude nucleic acid is dissolved in sodium hydroxide and is precipitated with hydrochloric acid and methylalcohol. Before reprecip-

itating the nucleic acid, we added about 10 mg  $(\text{NH}_4)_2\text{HPO}_4$  for each mg nucleic acid. By doing so, we dilute the free radioactive phosphate possibly present in the nucleic acid. If the crude nucleic acid carried before and after the precipitation 1 mg free phosphate, the free phosphate will be but 1/100 as active after precipitation as previously. This procedure is repeated several times, and each time inactive  $(\text{NH}_4)_2\text{HPO}_4$  is added to the alkaline solution.

The purification process entails a substantial loss of nucleic acid. However, it is not the total desoxyribose nucleic acid content of the organs in which we are interested, but the percentage of the desoxyribose nucleic acid content which is built up during the experiment, i.e. the rate of renewal of the nucleic acid molecules. We are interested in the activity of 1 mg nucleic acid P and not in the activity of the total nucleic acid present in the organs.

The purified nucleic acid is brought into solution by wet ashing.  $1/5$  is reserved for colorimetric P determination, while  $4/5$  are precipitated as ammonium magnesium phosphate; the activity of the precipitate is determined. The interpretation of the activity figures obtained is much facilitated if the precipitates, the activities of which are to be compared, have the same weight. To obtain this, a suitable amount (about 80 mg) of  $\text{Na}_2\text{HPO}_4$  is added to the solution before precipitating the ammonium magnesium compound.

An aliquot of the solution administered by subcutaneous injection is treated in the same way. If this "standard preparation" has, for example, 1/1000 of the activity administered and the nucleic acid fraction containing 1 mg P has 1/100 of the activity of the standard preparation, we find 0.001 per cent of the labelled P administered to be present in 1 mg nucleic acid P.

The weight of the male adult albino rats used varied between 250 and 320 g. They were kept on a normal diet. The labelled phosphate administered by subcutaneous injection had an activity corresponding to 3  $\mu$  Curie.

### Control of the Effectivity of the Purification Process.

To a crude nucleic acid fraction containing 200 mg nucleic acid we added 75,000 relative units of radioactive phosphorus ( $^{32}\text{P}$ ). Each time, decreasing amounts of inactive ammonium phosphate were added to the filtrate containing the nucleic acid. The amount of  $(\text{NH}_4)_2\text{HPO}_4$  added varied between 100 and 30 mg. After successive purifications, the following activity figures were obtained for the nucleic acid.

Number of purifications	Activity of fractions
0	75,000
1	—
2	34
3	9

The nucleic acid purified 3 times thus contained but 1/8000 part of the labelled phosphate added.

The successive purification of nucleic acid P from other than free P can be controlled in the following way. The specific activity of an aliquot of the nucleic acid P of the purified sample is determined. Another aliquot of the sample is dissolved subsequently and is reprecipitated as described on p. 002, but, without adding phosphate. The specific activity of the P of the precipitate obtained is then again determined. If no other phosphorus than nucleic acid P is present in the sample the specific activities determined should be identical. When controlling the purity of nucleic acid extracted from the liver and purified twice in the manner described above a further purification reduced the specific activity of the phosphorus obtained from the nucleic acid by 5.5 per cent.

Furthermore, we investigated whether the nucleic acid obtained is exclusively of desoxyribose type or contains also some nucleic acid of the ribose nucleic acid type.

To 7.6 mg active thymus nucleic acid dissolved in 1.0 cc 1/10 N. NaOH about 60 mg yeast nucleic acid, dissolved in 2 cc 1/10 N. NaOH were added. After precipitating but once, the nucleic acid, as described on p. 238, we reetermined the specific activity of the nucleic acid P. This was found to be 76 per cent of the specific activity of the value measured at the start of the experiment. A single precipitation sufficed thus to remove 96 per cent of the yeast nucleic acid added.

### Specific Activity of the free P of Different Organs.

The fact that greatly divergent figures are obtained for the specific activity of the nucleic acid fractions is due mainly to the highly different rate at which nucleic acid is formed in the different organs. It is, however, also partly due to the different rate at which the labelled phosphate penetrates into the cells in which the nucleic acid molecules are built up. To calculate the rate of formation of nucleic acid, it is thus necessary to know the specific activity of the "free" phosphate present in the tissue cells. It does not suffice to know the specific activity of the free P at the end of the experiment. We have to determine this magnitude at different times in order to arrive at a value of the average specific activity of the free P during the experiment. The results obtained are seen in Table 1 which contains data on the specific activity of the "free" P extracted from the organs. They are obtained on rats killed at different times. In the case

of the muscles, the free P was extracted from tissue samples taken from the narcotized rat. The tissue was placed in liquid air and extracted at once by grinding with 10 per cent trichloroacetic acid. This precaution has to be taken in order to avoid the decomposition of creatine phosphoric acid present in large amounts in the muscle. While, in experiments of long duration, as discussed below, the specific activity of the creatine phosphoric acid P does not differ from the specific activity of the free P, in experiments of shorter duration, however, large differences were found, and in these experiments a decomposition of creatine phosphoric acid prior to the removal of the free P would result in a lowering of the specific activity of the free P.

The specific activity of the creatine phosphoric acid was determined in the following way. After the removal of the free P as ammonium magnesium salt the filtrate was slightly acidified and heated for a very short time. The free P obtained by the decomposition of creatine phosphoric acid was then again precipitated as ammonium magnesium salt. While, after the lapse of one day or more, the specific activity of the creatine phosphoric acid phosphorus was found to be just as high as the specific activity of the "free" P present in the muscle tissue, after the lapse of 2 hours the ratio of the specific activities was found to be only 0.6.

Table 1.

*Specific activity of the free phosphate extracted from the organs.<sup>1</sup>*

Organ	Duration of the experiment in hours								Average value of the specific activity during the experiment
	2	5	8.5	13	25	50	72	94	
Plasma . . .	0.91	0.67	0.214	0.286	0.204	0.114	0.099	0.069	0.18
Liver . . .	1.61	0.95	0.42	0.59	0.17	0.15	0.10	0.10	0.25
Kidney . . .	1.10	0.78	0.43	0.57	0.18	0.16	0.096	0.10	0.24
Spleen . . .	0.77	0.67	0.31	0.43	0.19	0.16	0.11	0.10	0.21
Mucosa of the small intestine . . .	0.72	0.51	0.35	0.51	0.15	0.13	0.11	0.11	0.19
Muscle . . .	0.27	0.13	0.12	0.38	0.062	0.084	0.090	0.065	0.11
Testes . . .	0.13	0.12	0.086	0.14	0.062	0.079	0.072	0.06	0.080
Brain . . .	0.044	0.052	0.045	0.083	0.047	0.057	0.053	0.051	0.054

<sup>1</sup> Specific activity usually denotes the activity of 1 mg P in arbitrary units (often, the specific activity of the plasma P is taken = 100). The above specific activities denote the percentage of the <sup>32</sup>P administered present in 1 mg P.



## Specific Activity of the Nucleic Acid extracted from Different Organs.

The results of the determination of the specific activity of the nucleic acid phosphorus extracted from the different organs is seen in Table 2. I and II denote the values obtained in the first and second experiments, respectively. In each experiment the organs of 8 rats were pooled. The values shown in the last column indicate the percentage ratio of the specific activity of the nucleic acid P and the specific activity of the "free" phosphate P of the different organs.

Table 2.

*Specific activity<sup>1</sup> of the nucleic acid phosphorus extracted from different organs of 8 rats 4 days after the administration of labelled phosphate  $\times 1000$ .*

Organ	Specific activity $\times 1000$		Percentage ratio of the specific activity of nucleic acid P and free P (Percentage renewal)*
	I.	II.	
Small intestinal mucosa	12.7	15.4	59
Spleen . . . . .	5.93	6.24	23
Muscle . . . . .	1.05	1.42	8.8
Liver . . . . .	1.01	1.66	4.2
Testes . . . . .	0.83	0.97	10
Kidney . . . . .	0.60	0.67	2.1
Brain . . . . .	0.09	0.22	2.3

When calculating the figures of the last column, we have not taken the figures of the specific activity of the free phosphate P as stated in Table 1, but corrected these for the presence of labelled P in the extracellular space of the organs. The extracellular P is not utilized to build up nucleic acid, and we have to consider the ratio of the specific activity of the nucleic acid P and the specific activity of the cellular free P. The figures for the size of the extracellular space of the organs were taken from a paper by MANERY and HASTINGS (1939) and it was assumed that the labelled phosphate concentration of the extracellular fluid is identical with the labelled phosphate concentration of the plasma water. The correction for the presence of labelled phosphate in the extracellular space was largest for the testes, but even in this case

<sup>1</sup> Percentage of the <sup>32</sup>P-administered present in 1 mg P.

<sup>2</sup> When calculating the above ratio we must take into account that the nucleic acid has been extracted from the organs of 8 rats.

only 12 per cent of the value stated in Table 1. We did not correct the specific activity found for the free P of the brain in view of the uncertainty prevailing as to size and composition of the extracellular fluid of the brain. Therefore, it is possible that the rate of renewal of the brain nucleic acid is not slightly larger,

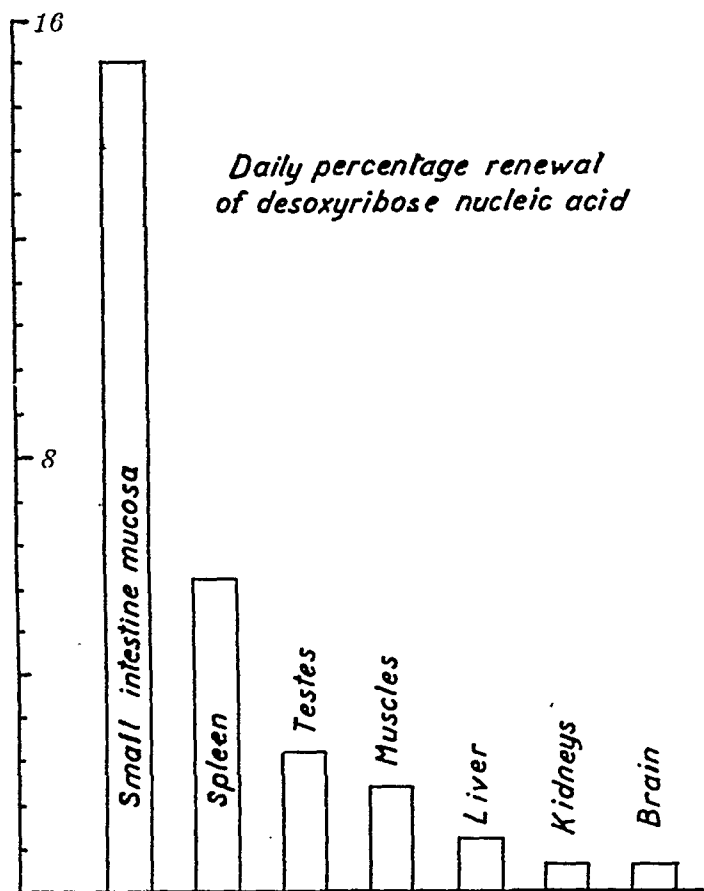


Fig. 1.

but smaller than the corresponding value found for the kidneys (cf. Table 2).

The percentage ratio of the specific activity of nucleic acid P and free P (the percentage renewal of the nuclei acid) in different organs is seen in Table 2 and Fig. 1. The highest percentage of new nucleic acid is found in the small intestine, while the lowest figure is shown by the brain. Remarkably low figures are found for the liver. In the course of 1 hour, a very large part of the acidsoluble P compounds and a few per cent

of the phosphatides present in the liver are renewed. Compared with these figures, the rate of renewal of nucleic acid in the liver is negligible.

The percentage ratio of the specific activity of the nucleic acid P and the free P indicates the percentage of new nucleic acid present, i. e. nucleic acid formed in the course of the experiment. We cannot state with certainty whether this new nucleic acid is formed in the organ in which it is found or transported from another organ in which it was built up. It would be conceivable that the nucleic acid molecules built up in the intestinal mucosa, for example, where we find by far the greatest rate of renewal of nucleic acid, reach the circulation and are deposited in the muscles. Information on this point can be obtained on the same line or on similar lines on which the origin of the phosphatides present in the yolk was investigated (HEVESY and HAHN 1938). The nucleoproteins are probably built up in the nuclei of the cells and not carried from organ to organ. The low new (labelled) nucleic acid content of the liver can be interpreted as an argument against the last mentioned interpretation. The liver takes up easily constituents present in the circulation and, if any organ takes up from the circulation nucleoproteins and thus nucleic acids, we would expect the liver to do so. The active nucleic acid content of the liver nucleic acid is, however, very low and this fact supports the view that the active nucleic acid molecules present in the liver are synthesized in this organ. The rate of renewal of the nucleic acid molecules in the liver may be identical with the rate of new formation of liver cells.<sup>1</sup>

The figures for the rate of renewal of nucleic acid in the organs of the rat found in this investigation are very much lower than those found for the renewal of nucleic acid or of "nucleoproteins" by different experimenters both in the organs of the rabbit and in the organs of the mouse. In the liver of the rabbit (HAHN and HEVESY 1940), for example, 6 per cent of the nucleic acid present were found to be renewed in the course of 11.5 hours. In the liver of the mouse (TUTTLE, ERF and LAWRENCE 1941), in the course of 6 hours, about 40 per cent of the "nucleoproteins" present were found to be labelled. In these experiments, the nucleic acid P and the "nucleoprotein P", respectively, con-

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<sup>1</sup> The rate at which liver cells are renewed is not known. While this rate may be smaller than the rate of renewal of nucleic acid in the liver cells, it can hardly be larger.

tained presumably some strongly active acid-soluble or phosphatide phosphorus, the presence of which was presumably responsible for the high values obtained for the rate of renewal of the nucleic acid and the "nucleoproteins".

### Amount of Nucleic Acid formed daily in the Different Organs of the Rat.

If we assume that the labelled desoxyribose nucleic acid found in an organ is synthesized in the organ in question, we can estimate from the data of Table 2 and the desoxyribose nucleic acid content of the organs the total amount of desoxyribose nucleic acid which is built up daily in the different organs. The desoxyribose nucleic acid of the organs of the rat is unknown. However, data are available on the total nucleic acid content of the organs of the rat. These data are given in Table 3. With the ex-

Table 3.

*Upper limit of the desoxyribose nucleic acid content of different organs of the rat.*

Organ	Desoxyribose nucleic acid content (mg per gm)
Muscle . . . . .	1.4
Heart . . . . .	1.4
Brain . . . . .	2.5
Kidney . . . . .	3.3
Testes <sup>1</sup> . . . . .	5.7
Mucosa of the small intestine . . . . .	5.1
Liver . . . . .	6.5
Spleen . . . . .	10
Thymus <sup>2</sup> . . . . .	30

ception of the figure stated for the nucleic acid content of the intestinal mucosa, they are taken from a paper by JAVILLIER et alia (1928). These workers state the nucleic acid P content of the tissue investigated; we multiplied their figures with 12 to arrive at the nucleic acid content. As no data were available for the nucleic acid content of the mucosa of the intestine, we determined the desoxyribose nucleic content of the mucosa small intestine by using DISCHE's method (1930) in a slightly modified

<sup>1</sup> Horse testes. (Javillier and Allaire 1926.)

<sup>2</sup> Horse thymus. For calf thymus, 36 was found. (Javillier and Allaire 1926.)

form, as applied by VOWLES (1940). This method is based on the fact that, when heating a solution of desoxyribose nucleic acid in the presence of diphenylamin, acetic acid and sulphuric acid, a violet colouring is obtained, the intensity of the colour being proportional to the concentration of the nucleic acid. As standard preparation we used a thymus nucleic acid preparation kindly presented us by Professor HAMMARSTEN. As the reaction used is not strictly specific for desoxyribose, the figure obtained has also to be considered an upper limit of the oxyribose nucleic acid content of the intestinal mucosa.

If the bulk of the proteins present were not previously precipitated, the colorimetric determination gave a higher value (6.9 mg). The same observation was made by VOWLES (1940).

The upper limit of the amount of desoxyribose nucleic acid built up daily in the different organs of the rat is given in Table 4.

Table 4.

*Upper limit of the amount of desoxyribose nucleic acid built up daily in the different organs of rats weighing on the average 275 gm.*

Organ	Weight in gm	Nucleic acid present in the organ in mg	Upper limit of desoxyribose nucleic acid built up in the course of a day in mg
Brain . . . . .	1.43—1.53	3.7	0.02
Kidney . . . . .	1.72—1.76	5.8	0.03
Testes . . . . .	1.93—2.57	12.8	0.34
Spleen . . . . .	0.86—0.81	8.3	0.48
Liver . . . . .	9.24—9.06	57.5	0.61
Mucosa of the small intestine . . . . .	4	21	3.0
Muscle . . . . .	111—109	154	3.4

As already mentioned previously, when calculating the above figures we assume that the nucleic acid found in an organ is built up in the organ. This assumption may not hold strictly, as the blood contains some nucleic acid which may have been carried from the organs into the circulation. However, this amount is but small. Assuming the rat blood to contain the same nucleic acid concentration as human blood which, according to JAVILLIER and ALLAIRE (1931) amounts to 0.3 mg per gm blood, the total amount of desoxyribose + ribose nucleic acid present in the circulation of a rat weighing 275 gm amounts to about 6 mg, thus about 1/50 of the total nucleic acid content of the rat.

### Summary.

Labelled phosphate is administered to adult rats by subcutaneous injection. After the lapse of 4 days, the rats are killed and the desoxyribose nucleic acid present in different organs is extracted. By comparing the activity of 1 mg desoxyribose nucleic acid P with the activity of 1 mg cellular inorganic P of the same organ, data on the percentage renewal of the desoxyribose nucleic acid present in the organs are obtained.

In the course of 4 days, only a minor part of the desoxyribose nucleic acid present in all the organs investigated is found to be labelled, i. e. renewed. The greatest daily renewal of nucleic acid (15 per cent) takes place in the mucosa of the small intestine. This is followed by the spleen (5.8 per cent), the testes (2.6 per cent), the muscles (1.9 per cent), and the liver (1.0 per cent). The lowest figures are shown by the kidneys and the brain (0.6 per cent).

The authors wish to express their sincere thanks to Professor NIELS BOHR for numerous facilities kindly put at their disposal.

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## On the Effect and Toxicity of Dimethyl Sulfide, Dimethyl Disulfide and Methyl Mercaptan.

By

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The literature contains very little data on the effect of dimethyl sulfide, dimethyl disulfide and methyl mercaptan on the living organism. A description of our experiments on the effect and toxicity of these substances should therefore be of interest.

*Dimethyl sulfide* is said to paralyze the sensibility (CURCI, 1895). About *dimethyl disulfide* no statements have been found in the literature, but the alkyl disulfides are said to be less toxic than the monosulfides (FLURY and ZERNIK, 1931). *Methyl mercaptan* is said to be considerably less toxic than hydrogen sulfide (REKOWSKI, 1893). The minimal lethal dose for rabbits is 169 mg of its calcium salt per kilogram of body weight, according to REKOWSKI, which is equivalent to 120 mg of  $\text{CH}_3\text{SH}$ . The minimal lethal dose for hydrogen sulfide seems to be of the same magnitude, however (MALOFF and co-workers, 1937, KEESER, 1930). The symptoms of methyl mercaptan intoxication are first stimulation of the respiration, and then paralysis.

The corresponding ethyl compounds are also little known. Thus 1 % *diethyl sulfide* in ether killed a dog twelve hours after the narcosis in one case. Hemorrhagic gastro-enteritis and hyperemia of the lungs was noted on autopsy (BOURNE, 1926). *Diethyl disulfide* causes headache (LEWIN, 1929), and animals can endure a concentration of 0.1 mg per liter for half an hour without any symptoms (FLURY and ZERNIK). *Ethyl mercaptan* is less toxic than diethyl sulfide (BOURNE). It has a moderately irritating (and vesicant) effect on the skin (OETTEL, 1936).

### Experimental.

White female rats, weighing 90–130 g, and starved 24 hours before the experiments, were used. In each experiment one rat was placed in a gas chamber of 7.6 liters volume (see fig. 1). After the observa-

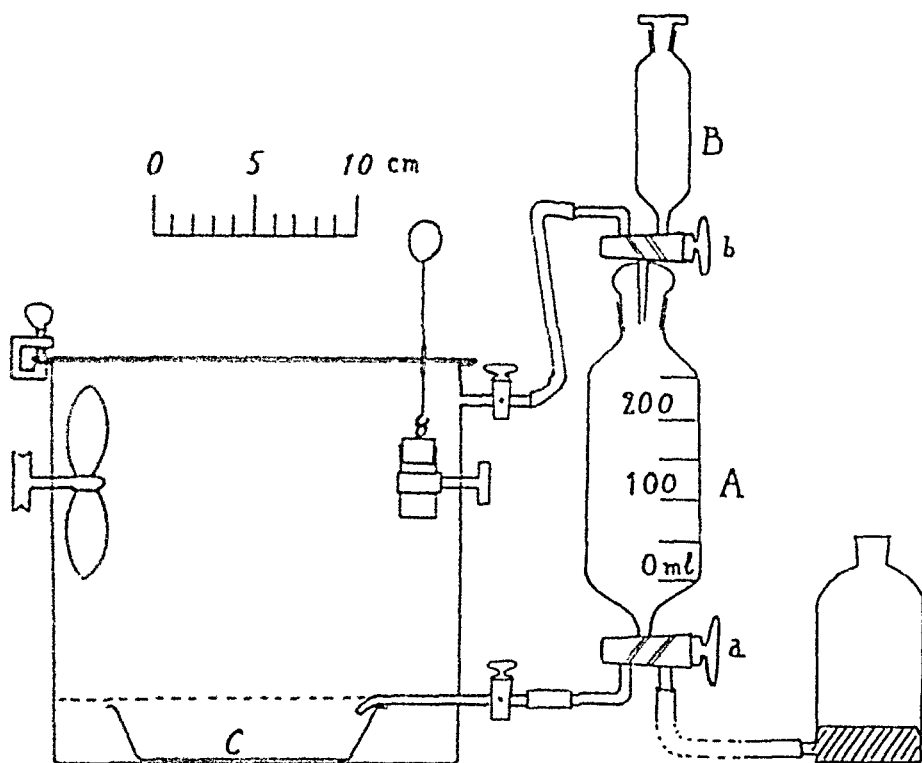


Fig. 1.

tion window had been hermetically closed, the substance to be tested was introduced from a calibrated gas burette or from a weighing bottle, the stopper of which could be unfastened from the outside. The time of the experiments was limited to 30–35 minutes. If the maximum carbon dioxide production is 60 mg per minute and kilogram of body weight (DONALDSON 1924), then the  $\text{CO}_2$  content in the chamber could reach 1.8 % by volume at the margin, which would hardly affect the results. During all the experiments a temperature of 22–25° was used.

The animals were observed during the whole treatment, and the surviving ones for 24 hours, after which they were killed by having their necks cut. The autopsy was complemented in some cases by *histological* examination of the lungs after fixation in 10 % neutral formaldehyde.

*Chemical analyses:* the concentration of the toxic agent was determined in samples taken with a special gas pipette (see fig. 1). This



pipette consists of a 300 ml. separation funnel, *A*, with a two way stopcock, *a*. The funnel is connected to another two way stopcock, *b*, and a smaller separation funnel, *B*, by a standard ground glass joint. The funnel *A* is graduated and the connections made as seen in the figure. At the start the pipette is filled with pure mercury. By letting out a suitable amount of mercury from the pipette into the chamber (bowl *C*), a corresponding amount of gas is driven over into the pipette. Stopcock *b* is then closed, and stopcock *a* is turned 180 degrees. By lowering the bulb the mercury is then drawn down to just below the stopcock *a* and this stopcock closed. By opening stopcock *b* the reaction mixture is sucked in from funnel *B*. The gas pipette is detached from the chamber and from the rubber tubing to the leveling bulb, and replaced by a new one. When the reaction is completed, the titration is made directly in the gas pipette *A*. For lubrication pure vaseline was used.

Mercaptan (and hydrogen sulfide) was determined iodometrically after absorption in sodium hydroxide, according to the reaction scheme:  $2\text{CH}_3\text{SH} + \text{J}_2 = (\text{CH}_3)_2\text{S}_2 + 2\text{HJ}$ . The iodine was produced from  $\text{KJO}_3$ ,  $\text{KJ}$  and  $\text{H}_2\text{SO}_4$ , and the excess iodine titrated with thiosulfate.

Dimethyl disulfide was determined according to the principle used by HARTNER and SCHLEISS (1936) for the estimation of glutathione. To test this method a solution of dimethyl disulfide in carbon tetrachloride was used, with a corresponding amount of carbon tetrachloride in the blank determination. It was found necessary to leave the samples for 4—8 hours in order to get complete transformation of the disulfide. According to the reaction scheme  $(\text{CH}_3)_2\text{S}_2 + 10\text{Br} + 6\text{H}_2\text{O} = 2\text{CH}_3 \cdot \text{SO}_3\text{H} + 10\text{HBr}$ , 1 mg of dimethyl disulfide should correspond to 1.06 ml of 0.100 normal  $\text{KBrO}_3$ . Values between 0.96 and 1.05 ml were found, with a mean value of 1.00 ml,<sup>1</sup> as seen from table 1.

In the experiments with dimethyl sulfide no chemical analyses were made.

## Results.

1. *Dimethyl sulfide*. The experiments show that dimethyl sulfide irritates the mucous membranes (secretion from the eyes and nose, difficulty in keeping the eyes open), and that it paralyzes the voluntary muscles and finally also the respiratory muscles. About 5 % by volume of dimethyl sulfide are necessary to kill a rat in 15 minutes. The results are summed up in table 2.

2. *Dimethyl disulfide*. The toxicity of this compound seems to be much greater than that of the monosulfide. The symptoms are similar, with signs of local irritation and a general effect resulting

<sup>1</sup> The discrepancy between the found and the calculated values for the  $\text{KBrO}_3$ -equivalent might be due to impurity of the dimethyl disulfide preparation used, but this possibility was not investigated, as the slight difference seemed to be of little importance to the evaluation of the results.

Table 1.

In every case: 10.0 ml of 0.100 n KBrO<sub>3</sub> + 5 ml of 2.5 n HCl + 8 ml of water + 1 ml of 3 n KBr + 0.7 ml of CCl<sub>4</sub> containing the dimethyl disulfide. After 6—8 hours 2.5 ml of saturated Na<sub>2</sub>HPO<sub>4</sub> + about 0.5 g of KI were added, and the free iodine titrated with thiosulfate with starch as indicator.

(CH <sub>3</sub> ) <sub>2</sub> S <sub>2</sub> mg	Equivalent amount of 0.100 n KBrO <sub>3</sub> ml	Ml of 0.100 n KBrO <sub>3</sub> per mg (CH <sub>3</sub> ) <sub>2</sub> S <sub>2</sub>
0	0	—
0.94	0.88—0.92	0.94—0.98
1.88	1.88—1.96	1.00—1.04
2.82	2.77—2.85	0.99—1.01
3.76	3.70—3.82	0.97—1.02
4.71	4.58—4.85	0.97—1.03
5.64	5.66—5.42	1.00—0.96
6.58	6.90—6.40	1.05—0.97

mean value 1.00

Table 2.

*Dimethyl sulfide.*

Concentration		Effects
mg/liter	% by volume	
3	0.11	No effect after 30 minutes.
14.5	0.56	Shut its eyes after 2 minutes, lay down after 10 minutes, let out after 30 min. Immediately lively.
34.6	1.3	Shut its eyes at once. After 5 min. slow respiration, after 10 min. also irregular, deep breaths, like sighs. Let out after 30 min. Instantly alert.
76	2.9	Acted like the foregoing rat. After 25 min. it staggered in attempt to get up. At 30 min. it lay on its side, did not get up by itself, respiration very fast and superficial, frequency about 200 per min. In 10 min. the rat seemed quite normal again.
80	3.1	After 5 min. staggered at attempt to rise. After 20 min. the rat lay on its side unable to get up. Let out after 30 min. Recovered in about 10 min.
140	5.4	After 2 min. fast, superficial respiration, the rat staggered and fell down on its side. After 5 minutes the dyspnea changed over into irregular, slow, deep respiration. Fluid bubbled from its nose. The respiration diminished gradually. Dead after 15 min. Autopsy: all organs smelled strongly of dimethyl sulfide, but no macroscopically visible changes were found.

Table 3.

*Dimethyl disulfide.*

Concentration		Effects
mg/liter	% by volume	
7.1	0.18	After 5 minutes disturbed respiration with occasional, deep convulsive breaths. Kept its balance when the chamber was tilted. Let out after 30 min., recovered immediately.
11.9	0.3	Respiratory rate immediately retarded. After 10 min. only deep, convulsive breaths, about 10 per minute. At 25 min. spasms, kicking movements with the legs. Recovery within 10 min.
12.9	0.33	The same symptoms in two rats on different days. The second rat showed no macroscopically visible changes on autopsy, but microscopically there were thickened alveolar walls, minute exudations in the alveoli and spotty infiltrations with macrophages.
19.2	0.49	After 5 min. slow, irregular respiration, eyes shut, secretion from eyes and nose. Convulsions. The rat lay on its side after 20 min. The respiration became faster and more superficial; let out after 30 min. The respiration gradually stopped. Dead after 35 min. Autopsy: blood-coloured fluid could be pressed out from the lungs. In another experiment with the same concentration the rat died after 15 min. only.
26	0.66	The respiration was at once disturbed. After 12 min. convulsions, the rat lay on its side. After 20 min. subsiding respiration. Dead after 25 min. Autopsy: petechial bleedings in the lungs.

in paralysis. There is some difference, however. The irritation seems to be stronger, resulting in convulsions and macroscopically visible ecchymoses in the lungs. The paralysis of the external respiration develops without foregoing paralysis of the locomotion. The results are evident from table 3. It appears that the concentration of dimethyl disulfide which kills a rat in about 15 minutes is only about one tenth of the concentration of dimethyl sulfide giving the same effect. This is remarkable, for alkyl polysulfides are stated to be less toxic than their monosulfides (see above).

3. *Methyl mercaptan.* This compound was used only for a few experiments. From the observations, which are summed up in table 4, it is evident, however, that the effect is that of general intoxication with more or less pronounced paralysis of the locomotory muscles and the respiration. Signs of local irritation were

Table 4.  
*Methyl mercaptan.*

Concentration		Effects
mg/liter	% by volume	
1	0.05	No effect in 30 minutes.
1.4	0.07	The rat seemed tired, but recovered instantly when taken out.
3	0.15	After 15 min. the rat could keep on its legs but obviously only with great difficulty. At the end of the experiment it could get up for a moment but then trembled all over its body. Recovery in 5 minutes. Microscopically: clustered changes of edema type: thickened alveolar walls, exudation in the alveoli containing blood cells.
20	1	Convulsions after 1 min. After 2 min. fast and superficial respiration. After 6 min. the rat lay on its side. After 8 min. respiration irregular. After 14 min. the respiration stopped. Autopsy: macroscopically, small bleedings in the lungs. Microscopically: alveoli stuffed with erythrocytes large areas, compensatory emphysema. Moderate amounts of serous fluid in the alveoli.

only found in the lungs at autopsy. The concentration necessary for killing a rat in 15 minutes seems to be of the same magnitude as for dimethyl disulfide.

### Discussion.

The investigated substances contain bivalent sulfur as a toxoforic group and the methyl radical, which in many substances has a distinct intensifying action on the toxicity (auxotoxic properties, see e. g. SARTORI, 1940, l. c. p. 28). Comparison between the effects of hydrogen sulfide, which effects ought to be those of the toxoforic group alone, and those of the substances in question shows good agreement on the whole. The chief symptoms are, on the one hand, local irritation of the mucous membranes of the respiratory tract and the eyes, and on the other a general toxic effect, probably blocking of essential endocellular enzymes (RODENACKER, 1927, BERSIN, 1940), which finds expression in palsies and occasionally in convulsions. As to the toxicity, however, there is a remarkable difference. According to FLURY and ZERNIK, and KOBERT, a concentration of 0.1 % by volume of hydrogen sulfide will kill a

rat, cat or rabbit in about 15 minutes. Of the investigated methyl derivatives, a 5 to 50 times greater concentration was necessary for the same effect on rats, as is evident from table 5. The volume percentage was chosen, as the concentration unit, because the concentrations could then be compared on a molecular basis. The concentration is also given in mg. per cubic meter, as this measurement is so often used in toxicologic literature. The toxicity values are likewise calculated on the basis of the two concentration units.

Table 5.

Compound	In 10—20 minutes fatal concentration		Relative toxicity	
	% by vol.	mg per 1000l.	10 % by vol.	152000 mg per 1000 l
Hydrogen sulfide . . . .	0.1	1520	100	100
Dimethyl disulfide . . .	0.5	20000	20	7.5
Methyl mercaptan . . .	1	20000	10	7.5
Dimethyl sulfide . . . .	5.4	140000	1.9	1.1

Apparently the toxicity of dimethyl disulfide and methyl mercaptan is about the same, which might be explained by the interrelation of the two compounds according to the equation  $(\text{CH}_3)_2\text{S}_2 + 2\text{H} = 2\text{CH}_3\text{SH}$ . The reason why dimethyl sulfide is so much less toxic is unclear. In hydrogen sulfide the toxoforic group acts as a sulfide or a  $\text{SH}'$  ion. This may possibly be true in the case of mercaptan too, but in dimethyl sulfide the methyl groups are so fixed to the sulfur, that saponification is very unprobable. In the dimethyl sulfide the toxic action of the toxoforic group is obviously limited through the firm binding of the methyl groups. Therefore, dimethyl sulfide shows a quite specific toxicity.

### Summary.

A number of experiments on the effects and toxicity of dimethyl sulfide, dimethyl disulfide and methyl mercaptan on rats are described. The effects of these sulfides are mainly the same as those of hydrogen sulfide. The toxicity, estimated from the concentration necessary to kill a rat in about 15 minutes, decreases in the order hydrogen sulfide — dimethyl disulfide — methyl mercaptan — dimethyl sulfide.

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## Propagation of Contraction in the Isolated Striated Muscle Fibre.

By

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Since HELMHOLTZ's measurement of the velocity of the nervous impulse, similar points of view have been transferred to excitation and propagation of contraction in muscle. According to E. WEBER (1846), contraction, indirect as well as direct, occurs simultaneously in all parts of the muscle, while BOWMAN (1840), and FICINUS (1836), basing their theories on microscopic observation, find a progressive propagation of the contraction over the fibre. The following decades of research concerned themselves mainly with propagation of the stimulation and contraction in total muscle, and were less interested in the propagation of excitation over the contractile substance itself — (BERNSTEIN 1871), HERMANN (1875), ENGELMANN (1897), BURDON SANDERSON (1898), HOFFMANN (1913), BETHE and HAPPEL (1923), FISCHER (1926), HAPPEL (1926) and ROSEMAN (1936). The so called "contraction wave", much discussed in this connection, cannot be regarded as an expression of the transference of excitation or contraction over the muscle fibre itself. The contraction-wave, measured in the directly or indirectly stimulated muscle, is only related to an out-of-phase activity, i. e. produced by a partly asynchronised stimulation of the different muscle fibres.

An analysis of the propagation of contraction, independent of the spread of the stimulus over the different parts of the muscle, can only be carried out by an examination of the single fibre.

Different indicators have been used to measure the propagation of activity over the fibre. WILSKA (1939), working on the propagation rate of excitation, measured by means of action potentials, found a propagation velocity of 1.5 m/sec. Measuring the mechanical tension developed by the two halves of the fibre, ASMUSSEN and LINDHARD (1935) found the *same* tension development in the two parts when the stimulation had reached a certain level. The experimental procedure used, however, allowed no recording of an eventual time difference in the tension development.

In the present experiments, the optically registered change in the cross striations (BUCHTHAL, KNAPPEIS and LINDHARD (1936), was used as an indicator of the propagation of the contraction process over the fibre. This registration has essential advantages over methods which use a time difference in tension development as indicator, as a number of errors, e. g. on account of differences in elastic after-effect, are thus avoided. Furthermore, the course of the tension development can be affected by differences in the cross-section of the fibre parts, as a larger or smaller part of the two halves of the fibre are passively stretched under, an only nominally, isometric contraction.

*A propagation of contraction will yield a progressive change of height of compartment, and especially a progressive change in the length of the anisotropic (A), and the isotropic (I) discs.*

1) If the contraction spreads over the fibre, the degree of stretch measured by the height of compartment ( $A + I$ ) will change progressively under the propagation. The changes in the height of compartment to be expected with propagation of contraction over the isometrically fixed fibre are very slight, as the tension — resulting from the contraction of part of a fibre only, and affecting the remainder of a still resting fibre — is small. Thus it can be seen that the changes in height of compartment can be judged only with great uncertainty.

2) On the other hand, the changes in proportion between the length of A and I which take place under contraction are considerable, and can be measured with sufficient accuracy. With simultaneous measurement of the time course of the changes in the length of the anisotropic and isotropic substance in two points of the same fibre under contraction, it is possible to determine the propagation rate, when the distance between the two points of measurement is known.



### Method.

To make possible a simultaneous microcinematography of the two points on the fibre, a fiber is bent around a glass rod in the form of a hairpin and placed so that the two parts of the fibre are alongside each other in the same microscopic field. The diameter of the glass rod is 0.5 mm. and the fibre is placed round the rod with a not-too-sharp bend, so that the fibre contents are not compressed.

The experiments are performed on single fibres of *m. semitendinosus* (*Rana esculanta* and *Rana temporaria*), which are prepared under the binocular microscope from tendon to tendon in an ice-cooled Ringer solution. Some control experiments are made with small fibre bundles. The pH of the Ringer solution, after a stream of 99 per cent  $O_2$  and 1 per cent  $CO_2$  has been passed through it for 5 minutes, is 7.3–7.4, and the pH value does not vary during the experiment. To ensure a suitable colloid-osmotic pressure, the Ringer solution contains, besides the

different salts and glucose, 6 per cent dialysed gum arabic., or 1.35 per cent Polyviol Am.

After preparation, the fibre is placed on a slide, around the above-mentioned glass rod (Fig. 1), and the two tendon ends are fixed by means of two movable clamps. A celluloid frame 0.45 mm thick, is fastened upon the slide and closed with a cover-glass, so that during the experiment, the fibre lies in a chamber filled with

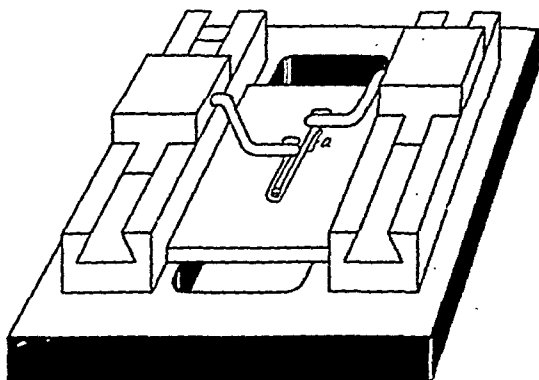


Fig. 1: Placing of the muscle fibre for simultaneous microcinematography of two points on the same fibre. a = origin of stimulation.

Ringer solution. The slide with the glass rod and metal clamps is attached to a bakelite plate which fits the mechanical stage of the microscope in horizontal position.

**Stimulation:** The stimulus is led to the tendon ends over the metal clamps, which fix the fibre on the slide. The stimulus is a rectangular current pulse with a duration of about 1 ms. The strength of the stimulus is chosen so that the contraction is maximal, without, however, causing an excitation travelling in the opposite direction. The chief current gradient is present in the fibre region a (Fig. 1). Furthermore, the results of the experiments clearly show that the origin of excitation is localised to this region of the fibre.

**Microcinematographic Registration:** The changes in the cross striation during the isometric twitch are recorded by a suitable number of pictures per second. A water-cooled mercury high pressure lamp (Philips

"Philora Sp. 500", brightness 33,000 Stilb, luminous flux 15,000 Lumen) is used. The light is collected by a water-cooled collector (2 lenses à 20 dioptr.) and sent through a constant temperature cooling trough of 10 cm thickness to a further collecting lens with focus on the slit of a rotating disc. The disc is 22 cm in diameter, and is driven by a synchronous motor making 3,000 revolutions per minute. In the disc are two slits, so that the picture frequency amounts to 100 pictures per second with an exposure below 0.4 ms.

The disc is placed immediately in front of the condenser of the horizontally placed microscope. (Optic: Zeiss Epi objective  $40\times$  (n. a. 0.65) and projection-eyepiece  $9\times$ ). For microphotographic recording a fall camera is used, with a film strip about 30 cm long. (Negative material: Agfa tone negative film "Special"). Rate of fall 1 m/sec.

The distance from the eyepiece to the film is 5 cm and the single microphotograph reduced by a rectangular diaphragm to a size  $10\times 20$  mm. Thirty pictures are recorded on one film strip.

*Measurement of the Negatives:* The microphotographs are measured on the negative under the microscope with an eyepiece micrometer with movable cobwebs, with a magnification of 12.5 times. The total magnification thus amounts to  $900\times$ . To obtain the compartment height, the length of 10 successive compartments is taken. Besides, 10 measurements of the A and I substance respectively are performed on each negative. The temperature in the muscle chamber is  $20^{\circ}\text{C}$ . and was determined thermoelectrically.

## Results.

### 1) Changes in Height of Compartment under Contraction.

The change in height of compartment under contraction differs according to whether the point of measurement is near to or far from the origin of the stimulus. When measuring *exactly at the point of stimulation*, a contraction followed by a passive stretching can be expected. This passive stretch has already been observed by GAD when working on total muscle (1879), and later more closely examined by FISCHER (1926). After stimulation, the fibre-part concerned contracts, and the subsequent stretch of the shortened fibre-elements is due to the spread of contraction over the whole fibre, and the height of compartment corresponds now to that in the resting condition. *In the middle of the fibre* one can expect, first, a passive stretching caused by the contraction in the vicinity of the point of stimulation, then a contraction, so that the height of compartment in this part of the fibre will be less than it was during rest, and finally, a passive elongation of these shortened elements due to the contraction of fibre parts

more distant from the point of stimulation. If the height of compartment is measured *at the distal end from* the point of stimulation, first a passive stretching is obtained, and later, a contraction to the original length when the whole fibre is in contraction.

*The differences in the time distance between moment of stimulation and changes in cross striation* can be used to indicate the position of the point of measurement relative to the point of stimulation, as this was selected at random. With short time distance, the point of measurement must be considered as situated near the point of stimulation, and far from it, should the time distance be long.

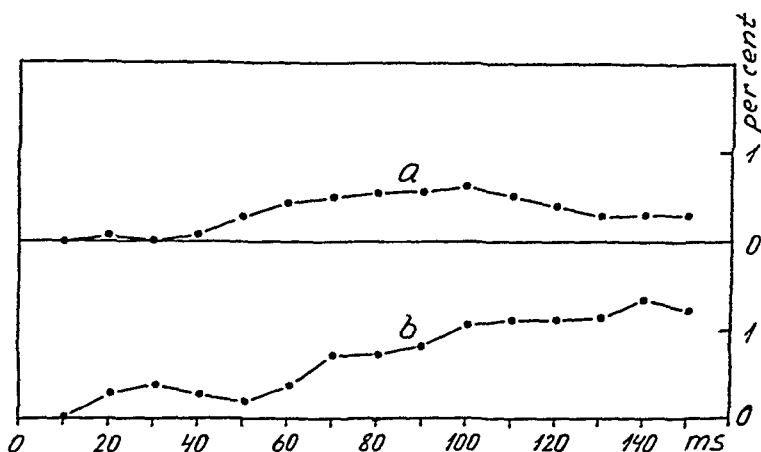


Fig. 2: Change in height of compartment during part of the course of an isometric twitch.

- a. near the point of stimulation,
- b. distant from the point of stimulation.

Abscissa: time in ms. Ordinate: change in height of compartment in per cent.

To estimate the changes of the compartment height under contraction, the material is divided into two groups, according to a short or long time distance respectively.

On account of the position of the stimulating electrode, in relation to the optical arrangement, it is difficult to measure directly at the point of stimulation, and the majority of measurements are taken around the centre part of the fibre, i. e. just before (short latent period), or just after (long latent period), the middle portion of the fibre.

An increase in height of compartment followed by a contraction is observed when measuring before the middle of the fibre (short time distance) (Fig. 2 a). For technical reasons, however, this increase cannot be followed through its whole course until the

height of compartment again equals, or becomes less than the original value.

When the point of measurement is distant from the point of stimulation (Fig. 2 b), the height of compartment shows larger variation, but here only the initial increase can be observed, as the subsequent contraction is not recorded, due to the limited number of pictures disposable. It is, however, possible that the subsequent contraction in both cases is somewhat masked by the viscous properties of the fibre, which retard the final consolidation.

When measured near the point of stimulation, the variation in length amounts to 0.5 per cent, while it is over 1 per cent far from the point of stimulation. This difference is to be expected, as near the point of stimulation a stretch is recorded which is quickly compensated by the contraction of the area where measurement takes place, while in the second case, only a stretch is recorded. Corresponding to the time difference found between the mechanical response and the changes in  $A$  in per cent of the compartment height  $\left(\frac{A}{A+I}\right)$ , where the latter varies more rapidly than the tension<sup>1</sup>, the change in length of height of compartment is not finished when  $\frac{A}{A+I}$  again has reached its original value. This retardation is due to the viscous properties of the fibre.

The *uncertainty* in judging the change in the height of compartment in the group with short distance from the point of stimulation, amounts to 0.2 per cent, and in the group with long distance, to 0.3 per cent. These values include measurement uncertainty as well as error on account of different points of measurement and individual variations, and the accuracy is clearly insufficient for a reliable measurement of propagation velocity. Much better results are obtained when using the change in length of  $\frac{A}{A+I}$  as an indicator.

## 2) Change in the Length of $A$ and $I$ in Simultaneous Measurements of two Points on the Fibre.

The maximal change in the length of  $A$  and  $I$  during a twitch of the isometrically fixed fibre is practically the same over the

<sup>1</sup> The experiments concerning this comparison will be described in a following paper.

whole fibre. The passive stretch of the parts distant from the point of stimulation does not measurably alter the change caused by contraction. At equilibrium length  $\frac{A}{A+I}$  amounts to 58 in the resting fibre, and to 50.5 in the maximally contracted fibre. We may, therefore, conclude that the propagation of the changes in cross striation proceeds without decrement over the whole fibre. The duration of the changes in  $\frac{A}{A+I}$  is about 0.1 sec. (20° C).

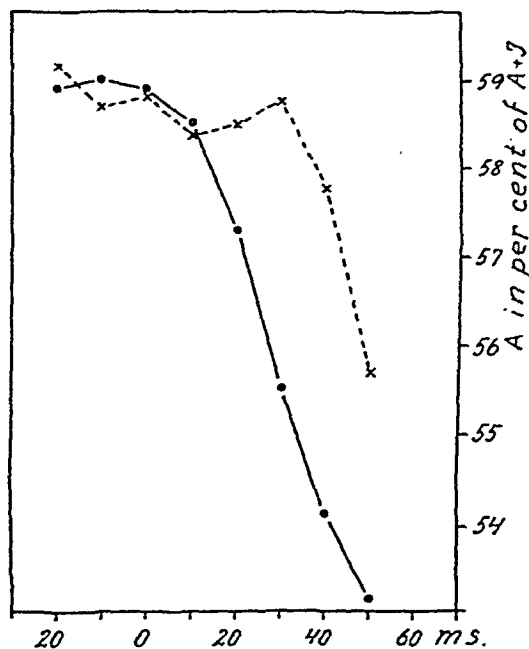


Fig. 3: Relative length of A and I substance in the isometrically contracted fibre from 20 ms before to 50 ms after stimulation.

●—●—● near the point of stimulation (a).  
 ×---×---× the fibre part lying 8 mm from (a).  
 Abscissa: time in ms. Ordinate: A in per cent of height of compartment.

Fig. 3 shows an example of the change in A's percentage of the height of compartment as a function of time for two points of the same fibre under contraction. The distance between the two points of measurement is 8 mm and the points on the curve are mean values of four experiments on the same fibre.

On the basis of the change in cross striation for the two halves of the fibre during contraction, a time difference of about 23 ms is found for the course of changes in

$$\frac{A}{A+I}$$

The error in the determination of the state of the cross striation at a given moment is, for the single point consisting of 10 measurements of A and I in the mean 1.30 per cent of the height of compartment. In the present curve, where each point is determined by 40 measurements, the error amounts to 0.65 per cent. From this, the corresponding time uncertainty is calculated. The variation in time of A's percentage in the height of compartment is 3.7 per cent in the course of 20 ms corresponding to 0.185 per cent per ms. On the other hand, the time corresponding to 1 per cent change in A's part of height of compartment is 5.4 ms.

With an error in  $A$  of 0.65 per cent, the time uncertainty becomes  $\frac{0.65 \times 5.4}{1} = 3.5$  ms.

In the range which is used to measure the time difference, the two curves have 3 pairs of points which are used directly to measure the parallel displacement (Fig. 3). When the error in determination of the single point is 3.5 ms, the corresponding uncertainty in determining the difference between the two values with the same uncertainty is:

$$\sqrt{3.5^2 + 3.5^2} = 3.5 \cdot \sqrt{2}.$$

When using three pairs of points, the resulting error amounts to  $\frac{3.5\sqrt{2}}{\sqrt{3}} = 2.9$  ms or 12.5 per cent of the time difference.

This error refers to the points each determined by 40 measurements. The error in the remainder of the material where each point is determined by 10 measurements, is 5.7 ms.

Revising the total material where the time difference is determined by the position of the inversion points of the two curves, we find an error in the propagation time of 14 per cent, corresponding to an error in time of about 3 ms. This shows that the error in estimating the point of inversion is less than the error in judging the parallel displacement of the curves.

The uncertainty found, which is the result of the error in measurement and the variation in the rate of propagation, is, with our knowledge of the error in measurement, mainly due to the latter. There is, therefore, no reason for supposing that the velocity of propagation itself varies essentially with the fibres examined.

The whole material is put together in Table I. The experiments are performed with different distances between the points of measurement (4.5–8.0 mm) and a corresponding phase difference of 13 to 25 ms is found, while the resulting rate of propagation is practically unchanged:  $0.32 \text{ m/sec} \pm 0.016$  ( $20^\circ \text{ C}$ ).

Table I.

<i>Experiment: Number:</i>	<i>Distance between Points of Measurement mm</i>	<i>Phase Difference ms</i>	<i>Velocity of Propagation: m/sec</i>
32 B	4.5	15	0.30
32 E	4.5	19	0.24
32 G	4.5	16	0.28
35 D	4.6	13.5	0.35
53 E	8.0	21	0.38
53 G	8.0	25	0.32
53 H	8.0	23	0.35
53 J	8.0	23	0.35
Mean Value:			$0.32 \pm 0.016$

### Discussion.

A so-called propagation rate of between 2.0 and 4.4 m/sec has been claimed for the contraction of total muscle. Compared with this, a very small propagation velocity is found in a single frog muscle fibre.

It must, however, be considered that stimulation of a non-curarised muscle always excites the nerve or the nerve endings, giving thereby a quicker spread of excitation, which is determined by the velocity of the nervous impulse, and in this case, as pointed out by LINDHARD (1931), it is inappropriate to define it as a wave of contraction.

A stimulation which affects one end of the muscle can in a completely curarised muscle only be transmitted to the distal end of the fibres directly stimulated. Naturally, as fibres are of such varying lengths, only a part of the fibres can reach the stimulated end of the muscle. If the whole muscle is to be stimulated, the current gradient must, at its weakest point, be above the threshold values of all fibres, and the contraction will be initiated at different places in the muscle at the same time. The speed of transmission of this so-called contraction wave will thus be determined, in the non-curarised muscle by the velocity of the nervous impulse, and in the curarised muscle by the difference in the latent period or in the gradient of the stimulation current whereby different points of a single fibre may be stimulated. Many points can be stimulated simultaneously with a high current gradient, but only few with a small, resulting in a quick development of contraction in the first case, and a slow development when the gradient is low. It may thus be concluded that the experimental results from total muscle cannot be considered as an expression for the propagation of contraction over the contractile substance. It is therefore impossible to correlate the propagation velocity found in the single fibre (0.32 m/sec) with the values obtained on total muscles. As controls with small fibre bundles have shown, the single fibre value is not altered by the isolation as such.

The propagation of the contraction over the fibre takes a certain time, and brings about that we never find simultaneous changes in the mechanical properties in the fibre as a whole, because the changes must have a certain transition period, corresponding at the lowest to the propagation time.

This finds an expression in the course of tension development as a function of time. When it is supposed that the individual fibre element contracts instantaneously and sharply, i. e.  $\frac{d \text{ tension}}{d \text{ time}}$  is constant from the moment tension begins to develop, a sudden change of tension should be expected in the resulting tension curve for the total fibre. However, in the tension-time diagram for the total fibre, a smooth transition is generally seen, and the

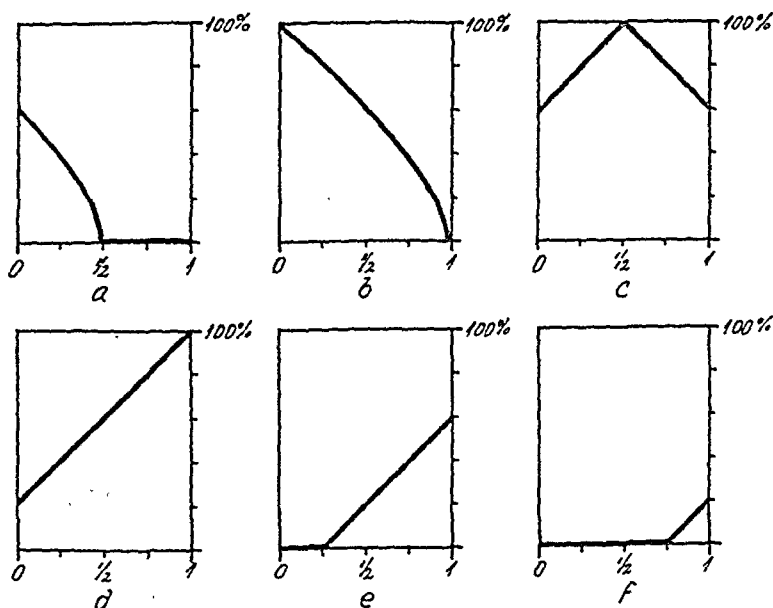


Fig. 4: Cross striation as a function of distance from stimulated point.

$a = 20$  ms,  $b = 40$  ms,  $c = 60$  ms,  $d = 80$  ms,  $e = 100$  ms and  $f = 120$  ms after stimulation.

Abscissa: distance from stimulated point in arbitrary units. Ordinate: change of  $\frac{A}{A+I}$  in per cent of the maximal change of  $\frac{A}{A+I}$  ( $= C$ ).

transition time from the beginning until  $\frac{d \text{ tension}}{d \text{ time}}$  is constant, i. e. until the tension development becomes linear, is about 10–15 ms. In a fibre 1 cm long, this should correspond to a propagation velocity of 1.60–1.00 m/sec. But the actual transition period is longer; it is not noticed because of the extremely low tension at the beginning of the contraction. As this concerns a passive stretch of a fibre, whose main part is at rest, the measurement of the tension development in this range is subject to a considerable inexactitude.



The more rapid the velocity of propagation, or the shorter the fibre, the shorter a propagation time and transition period can be expected. *A quickly working muscle must have a low propagation time, i. e. high propagation velocity or short fibres.*

From the rate of propagation (0.32 m/sec) and the time course of the changes in cross striation for a single fibre element, as is schematically shown in curve I, Fig. 5, it is possible to figure out the condition of the cross striations at any one moment, and at

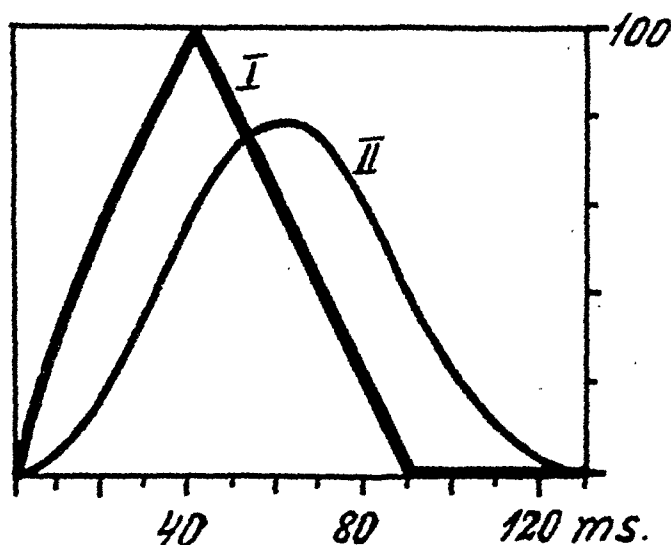


Fig. 5: Change of  $\frac{A}{A+I}$  during an isometrical twitch.

- I. for a single compartment.
- II. average for the entire fibre.

Abscissa: time in ms. Ordinate: change of  $\frac{A}{A+I}$  in per cent of the maximal change of  $\frac{A}{A+I}$  (= C).

any distance from the point of stimulation. An example is seen in Fig. 4, where the propagation time over the total fibre is put at 40 ms (corresponding fibre length is 13 mm). The curves a—f show schematically the condition of the cross striations of the fibre 20, 40, 60, 80, 100 and 120 ms after the stimulation. The abscissae correspond to the distances of the fibre element in question from the point of stimulation and the ordinates represent the state in the cross striation in proportion to the resting fibre in percentage of the maximal change of the ratio  $\frac{A}{A+I}$  denoted by C (at maximum contraction  $C = 100$ , at rest  $C = 0$ ).

Diagram (a) shows the condition of the cross striation 20 ms after stimulation. Nearest to the point of stimulation, C is 60, in that this point has been excited in all the 20 ms. A quarter of the fibre's length away from the point of stimulation, C is 45 per cent. Here the fibre has only been stimulated for 10 ms and half its length from the point of stimulation the fibre is about to go into contraction, the propagation time for the contraction being 20 ms for this section. The rest of the fibre is still in a resting condition, and is stretched about 1 per cent. The change of C brought about by this stretch is only 0.1 per cent, and therefore not measurable. 40 ms after the stimulation (b), C nearest the point of stimulation amounts to 100, and the last element of the fibre is just beginning to contract, corresponding to a propagation time of 40 ms over the whole fibre. Diagram (c) shows the condition of the cross striation 60 ms after stimulation. The middle part of the fibre has, at this moment, a C of 100. C decreases for the first half — where the contraction has passed its maximum — and increases for the second half. 80 ms after the cessation of the stimulation (d), the last element of the fibre has a C of 100 per cent (maximal contraction), while C decreases for the remainder. In Fig. 4 (e), representing conditions 100 ms after stimulation, the first quarter of the fibre is again at rest, and C decreases towards 0 for the remainder of the fibre. 120 ms after stimulation, (f), the first three-quarters of the fibre are at rest, and after a further lapse of 10 ms, the entire fibre will be at rest.

In Fig. 5, the state of the cross striations in a single compartment is compared with the average change in  $A + I$  for a whole fibre under contraction as a function of time. In the curve for a single fibre element (curve I), C increases steeply, continues straight, and reaches its maximum after a lapse of 40 ms. After this, C decreases linearly, and after a further 50 ms reaches its original value. The curve is constructed on the basis of the direct measurements of A and I.

Curve II shows the average C for a total fibre with a propagation time of 40 ms (fibre length 13 mm). The individual points of the curve are obtained by integration of the curves in Fig. 4. It is seen that C as a maximum for the total fibre only amounts to about 80 per cent, and that the curve is less steep. Furthermore, the changes take 40 ms longer than for the isolated element. Compared with the isometric tension development in the mechanical response, the steepness is more pronounced at the beginning

on account of the high viscous resistance against stretching of the individual fibre element. The example chosen is valid for a propagation time of 40 ms corresponding to a propagation distance of 13 mm. When a 13 mm fibre is stimulated indirectly over the nerve, and the end plate is supposed to be located in the middle of the fibre, the propagation distance for the two halves of the fibre is naturally halved, and a steeper course can be expected for the mechanical response.

BROWN and SICHEL (1936) think that "all the contractions previously obtained with fine electrodes must be classed as local contractions", and try to avoid local contraction and a propagation by a special method of stimulation. Instead of stimulating by a current applied in a longitudinal direction, the fibre is placed in an approximately transverse electric field. The present experiments show that contraction is propagated when the fibre is stimulated by a current with a gradient in longitudinal direction. It seems doubtful if the method of stimulation applied by BROWN and SICHEL insures a simultaneous contraction of the entire muscle fibre, as experience has shown that it is difficult to stimulate with a current, the resultant of which goes transversely to the axis of the fibre. Currents with components longitudinal to the axis of the fibre will only be present at the ends of the plate electrodes, and it is, therefore, highly probable that the excitation and contraction are initiated at the ends of fibre only (from the edges of the electrode plates), and that the stimulus is spread from both ends towards the middle of the fibre.

The propagation time of the contraction will have considerable influence on the determination of the *latent period* of the changes in cross striation during contraction.

In experiments which will be described in detail in a following paper, and which induced the present experiments, considerable

variations in the latent period of the changes in  $\frac{A}{A+I}$  were found,

while the latent period of the mechanical response in the various experiments only showed slight deviations. The accuracy in determining both latent periods amounts to 2 ms in the experiments in question. If we suppose, as seems natural, a causal relation between the changes in length of the A and I substance and the state of contraction, and further, a synchronous appearance of the contraction over the entire fibre, these large variations in the latent period are inexplicable. On the other hand, the

deviations are to be expected when the contraction does not occur over the whole fibre at the same time, but propagates over it. As the point of measurement for registering the change in length of the cross striation was chosen at random in every experiment, a change in its localisation in relation to the origin of the stimulus will cause a difference in time, which becomes longer with an increase in time of propagation.

The velocity of propagation, which amounts to 0.3 m/sec for the single fibre, is essentially lower than the rate of spread found for action potentials. If the values of WILSKA (1939) are applied, action potentials spread five times more quickly than the changes in cross striation.<sup>1</sup>

Supposing similar electrical and mechanical conditions for the *single fibre element* with regard to tension development and latent period, it must be expected that the potential accompanying a contraction propagates at the same rate as the optically measured changes in the fibre. In so far as WILSKA's findings are correct, this is not the case for the action potentials, and it must therefore be concluded that the *spread* of action potentials — corresponding to the excitation potential (E—F potential) differentiated by BUCHTHAL and LINDHARD (1937) — has no *direct* connection with *propagation* of contraction.

### Summary.

The propagation of contraction over the isolated muscle fibre is measured by the changes in the cross striation, i. e. shortening of the anisotropic (A), and lengthening of the isotropic (I) substance being used as indicator. Simultaneous microcinematography (100 pictures/sec.) of two points of the isometrically placed fibre shows a phase difference in the course of the changes in cross striation, which corresponds to a propagation rate of 0.32 m/sec  $\pm \pm 0.016$  (20° C). The change in the cross striation is transmitted through the fibre without decrement. Dependent on the distance between point of measurement and point of stimulation, an in-

<sup>1</sup> BUCHTHAL and LINDHARD (1937, 1939) have not denied a spread of the potential accompanying excitation, as is quoted in error by WILSKA (1939), but have expressly referred to the potential as "spreading over the fibre as a wave" (1939 p. 106), which, however, must not be confused with the so-called contraction wave in the whole muscle. It is still unexplained, however, whether it concerns a decrementless propagation or an electrotonic spread of potential (BUCHTHAL 1937).

crease in height of compartment is found so long as the contraction has not reached the fibre elements under observation.

The rate of propagation of contraction is essentially lower than that of the spread of action potential (excitation potential). The influence of propagation velocity on the course of the mechanical tension developed, and on the latent period, are discussed.

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# A Method of Correcting for Counting Losses in Geiger-Müller Counters with Special Reference to the Geiger-Müller Counter Arrangement Described by Hilde Levi (1941).

By

IB HOLM-JENSEN.

Received 11 January 1943.

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A GEIGER-MÜLLER counter arrangement mainly designed in accordance with the description given by HILDE LEVI, has been used in this laboratory during the last two years. We found that it was possible to get reliable results up to 500 counts per minute or even more. The limiting factor is the mechanical recorder, which was found to require frequent readjustments, when the impulses registered exceeded about 500 per minute for more than a very short time.

However, when the counting rate exceeded about 150 impulses per minute, and it is often important to be able to count such strong preparations, we found it necessary to apply a more accurate correction for counting loss, than that employed by HILDE LEVI, who carried out the correction from the following formula:

$$N = N_{\text{obs}} + \frac{N_{\text{obs}}^2}{k} \dots \dots \dots I$$

where  $N$  was the actual number of incoming impulses per minute and  $N_{\text{obs}}$  the number of impulses recorded per minute. The constant  $k$  was determined empirically and was found to be 1,500.

As the statistical fluctuations in the number of disintegrations

from a radioactive substance is about  $\sqrt{y}$ ,  $y$  being the number of impulses considered, and as a total of 10,000 impulses is practically the maximum counted from any sample in biological work, the probable fluctuation will be  $\cong \frac{\sqrt{10,000}}{10,000} \cdot 100 = 1$  per cent.

It is therefore desirable to keep the error due to approximate correction for counting losses below this figure in order to obtain the highest possible accuracy.

The counting losses are caused by the finite recovery time of the GEIGER-MÜLLER circuit, the amplifier and the recorder, because impulses arriving during these recovery periods are not transmitted respectively not recorded.

The recovery times of the GEIGER-MÜLLER circuit and of the amplifier are negligible compared with that of the mechanical recorder, and need not be considered at the counting rates in question.

Until recently it was customary to compute the actual incoming impulses per minute from the following formula (VOLZ, 1935):

$$N_{\text{obs}} = N \cdot e^{-N\tau} \dots \dots \dots \text{II}$$

where  $\tau$  is the recovery time.  $N$  and  $N_{\text{obs}}$  are here used in the same sense as in equation I. However, RUARK and BRAMMER (1937) stressed the fact that the above equation is based upon the assumption that an impulse in order to be registered must be preceded by an interval  $\tau$  during which no impulses arrive. In other words, if another impulse comes in during the recovery period this (even if it is not registered) should re-excite the recorder and add to the recovery time and thereby cause a further increase in counting loss. When, on the other hand, a recorder is completely unaffected by additional impulses coming in during the recovery period  $N$  may be computed from a much simpler equation.

$$N_{\text{obs}} = \frac{N}{1 + \tau \cdot N} \dots \dots \dots \text{III}$$

The deduction of RUARK and BRAMMER is as follows: Consider the interval  $\tau$  following the arrival of an impulse. The average number of impulses lying in this interval is  $\tau \cdot N$ , but there are  $N_{\text{obs}}$  such intervals per minute. Thus  $N = N_{\text{obs}} + N_{\text{obs}} \cdot N \cdot \tau$  which gives the above equation.

Equation II may be written

$$N_{\text{obs}} = \frac{N}{1 + \tau N + \frac{\tau^2 N^2}{2!} + \frac{\tau^3 N^3}{3!} + \dots}$$

thus it is seen that there is only a minor difference between formula II and III if  $\tau \cdot N$  is small. The difference, however, is by no means negligible at our counting rates.

Example:

$$\text{If } N = 500 \text{ and } \tau = \frac{1}{1,500}$$

equation II will give

$$N_{\text{obs}} = 500 \cdot e^{-1/3} = \frac{500}{1.396} = 358$$

whereas equation III will give

$$N_{\text{obs}} = \frac{500}{1 + 1/3} = \frac{500}{1.333} = 375$$

The difference is 5 per cent.

It may be quite difficult to find out whether a recorder should be considered as belonging to one type or the other from theoretical considerations regarding its construction; but the problem can easily be solved experimentally by isolating the grid of the thyatron valve; the recorder will now behave as if it received impulses transmitted from a radioactive source of infinite strength.

From equation II is derived

$$\text{if } N \rightarrow \infty, \lim N_{\text{obs}} = 0$$

and from equation III

$$\text{if } N \rightarrow \infty, \lim N_{\text{obs}} = \frac{1}{\tau}$$

Thus, when the number of impulses received approach infinity, a recorder of one type will stop recording, whereas a recorder of the other type will work at its maximum speed.

The values of  $N_{\text{obs}}$  thus determined for the mechanical recorder in question are found to be 1,200 to 2,000, depending on the adjustment of the recorder, and of the strength of the anode current in the thyatron circuit.



The value of  $N_{\text{obs}}$  obtained in the above test is about 10 per cent smaller than  $\frac{1}{\tau}$  found as shown below. So we may conclude that our counter is practically unaffected by impulses arriving during the recovery period, and for our purpose we may safely apply the correction formula III.

We have also tested counters of different design which clearly belonged to the group to be corrected by equation II.

The constant  $\tau$  is readily determined in the same way as used by HILDE LEVY for determination of her constant  $k$  which is seen to be nearly equal to  $\frac{1}{\tau}$  when  $N$  is small.

$\tau$  can also be computed from the following equation derived from equation III:

$$\tau = \frac{n x_n - x_1}{(n - 1) x_n \cdot x_1}$$

where  $x_1$  and  $x_n$  are countings per minute from the decay of some suitably shortlived radioactive substance, e. g. ThB with a half period of 10.6 h,  $n$  being the ratio between the corrected counting corresponding to  $x_1$  and  $x_n$  which is computed from the time interval considered and the known length of the half period. E. g. if the time interval considered is equal to the half period,  $n$  will be equal to 2 and

$$\tau = \frac{2 \cdot x_2 - x_1}{x_2 \cdot x_1}$$

for this computation it is best to use readings from a smoothed decay curve.

As a standard of the radioactive preparation used in our experiments is always counted regularly during the decay, and as this standard for practical reasons is generally chosen at least of equal activity to the strongest sample, we usually obtain the necessary facts to recalculate the length of our recovery period and thus check the adjustment of our counter. When  $\tau$  is computed the correction for counting loss is easily carried out by equation III which may be written

$$N = \frac{N_{\text{obs}}}{1 - \tau N_{\text{obs}}}$$

or

$$N = N_{\text{obs}} + \frac{\tau N_{\text{obs}}^2}{1 - \tau N_{\text{obs}}}$$

$\frac{\tau N_{\text{obs}}^2}{1 - \tau N_{\text{obs}}}$  thus being the counting loss.

We found it most convenient to carry out the correction in the following way:

III is written

$$\frac{N_{\text{obs}}}{N} = 1 - \tau \cdot N_{\text{obs}}$$

The straight line representing the relation thus given between  $\frac{N_{\text{obs}}}{N}$  and  $N_{\text{obs}}$  is drawn. (Fig. 1).

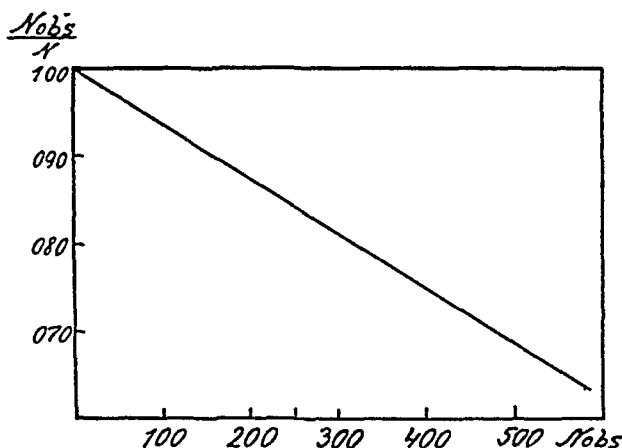


Fig. 1.

$$\frac{N_{\text{obs}}}{N} = 1 - \frac{1}{1,600} N_{\text{obs}}$$

When  $N_{\text{obs}}$  is given the corresponding value of  $\frac{N_{\text{obs}}}{N}$  is found on this line and  $N$  is computed from

$$N = N_{\text{obs}} : \frac{N_{\text{obs}}}{N}$$

In this way the computations are reduced to a minimum.

I am greatly indebted to Mr. H. K. ROLVED, Civil Engineer of the Telephone Company Automatic in Copenhagen for kindly placing at my disposal mechanical recorders of different design.

### Summary.

The theoretical basis of correcting for counting losses in a simple GEIGER-MÜLLER counting arrangement is outlined and a convenient procedure for carrying out the correction is presented.

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## The Determination of Ascorbic Acid in Biological Fluids.

By

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The methods generally used for the determination of ascorbic acid are based on the reducing property of this substance and are usually carried out as titrations by means of an oxidation-reduction indicator. Curiously enough the two indicators used occupy very different positions on the oxidation-reduction scale, methylene blue having a normal potential ( $E'_0$ ) of  $+0.011$  or very close to that of the ascorbic acid system itself, while  $E'_0$  for 2—6 dichloroindophenole is  $+0.219$ . The potential of methylene blue is in fact so close to that of ascorbic acid itself, that methylene blue cannot be used for the titration of ascorbic acid directly, but only under the activating influence of strong light. The degree of activation or change in the potential depends on the intensity of the light and it may be difficult to secure conditions, rendering the results of various authors comparable. To the use of dichloroindophenole it has been objected that while the large difference in the potential of the two systems guarantees the complete titration of the ascorbic acid, it involves at the same time the risk that other substances having potentials situated between the two systems may interfere and be titrated more or less, thus giving rise to too high results. The reaction velocity of the process between the dye and most of the interfering substances seems, however, to be much slower than that of the process between the dye and ascorbic acid, though it has been proved to be so only in some cases. The difficulty of the interfering substances has to some

extent been overcome by taking advantage of this fact, partly by performing the titration at a  $p_H$  where the interfering substances are least active, partly by considering the endpoint as reached when the colour of the oxidized dye is stable in the solution for a short time, by which procedures the slower reacting substances are to some extent eliminated.

Methods have been described, however, (MINDLIN and BUTLER, 1937; BESSEY, 1938; EVELYN, MALLOY and ROSEN, 1938), where the difference in the velocities of the reactions have been used in a somewhat different manner to distinguish between ascorbic acid and the substances reacting more slowly. The principle of these methods is that the solution to be analysed for ascorbic acid is mixed with a known amount of dye and the progress of the decolorization followed by means of an electrophotometer. The resulting curve is used for an extrapolation to zero time for the slowly reducing substances. The methods described in this paper are developed on this principle but differ in details, notably in that no precipitation of the proteins of blood serum is performed.

Because of the objections, which may be raised against the use of both methylene blue and dichloroindophenole, it was tried whether other indicators having potentials intermediate between these two systems were more suitable. The indicators tested were thionine (+ 0.063), toluylene blue (+ 0.115) and thymol indophenole (+ 0.174). The potential of thionine proved still to be too close to that of ascorbic acid, so that just as with methylene blue activation by light was necessary to bring about the reaction. Both toluylene blue and thymol indophenole reacted readily with ascorbic acid; the velocity of the reaction proved, however, to be slow in both cases. The result of this is that the decolorization curves obtained are drawn considerably out which makes it difficult to distinguish between ascorbic acid and the other substances. As nothing seemed to be gained by the use of these indicators dichloroindophenole was adhered to.

An essential feature in the methods is the use of an electrophotometer. The electrophotometer used in this study (Fig. 1) has been constructed in the workshop of the laboratory and incorporates some details, which differ from the commercial types. It is of the one-cell type and a constant light source is consequently imperative. This has been obtained in two different ways, one of which is by means of a "Stabilovolt" aggregate, which reduces the city 220 volt d. c. to a constant voltage of 110 volt, which

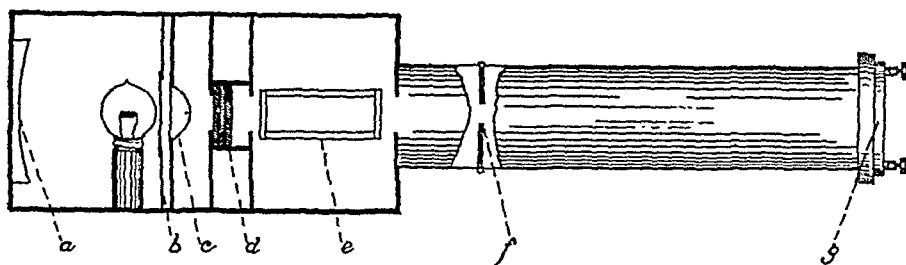


Fig. 1. Electrophotometer.

a mirror, b heat absorbing filter, c lens, d colour filters, e absorption cell, f iris diaphragm, g photocell.

is used to feed a 15 watt bulb. In this laboratory this arrangement has proved sufficiently constant as the variations in the current has been of the order of 1 pro mille, but in an apparatus working in another laboratory the stabilization was not effective enough, probably due to unusually large changes in the voltage of the electricity supply of the building, which housed a large industrial plant. The other stabilization method, which has proved the most satisfactory, consists in charging a storage battery with exactly the same current as that drawn from it for the bulb of the photometer. In this arrangement a 6 volt, 3 watt bulb has been fed from a battery, which is being constantly charged from the city 220 volt a.c. system through an arrangement consisting of an oxyde valve, a transformer and a resistance allowing the charging current to be regulated to correspond to that of the bulb.

The light from the bulb is collected into a narrow band by means of a cylinder lens (a front lens from an electric hand lamp may suffice) in front of which is placed a heat absorbing colloidal gold glass plate so as to avoid undue heating of the colour filters and the fluid sample. The colour filters which are combined from a set of filters from Schott und Genossen, Jena, are placed in a holder between the lens and the absorption cell. The combination used for the ascorbic acid determinations consists of 2 mm thickness of V.G. 9 and 4 mm of B.G. 18 which is suitable for the measurement of dichloroindophenole in the acid range. To the absorption of these filters that of the blue tinged heat filter is of course added which, however, makes but little difference in the results.

All electrophotometric methods involve a blank determination carried out with an absorption cell filled with water or with the

solvent. This blank determination is the more important the weaker the colour to be measured. The use of a separate absorption cell for the determination of the blank involves, however, the danger that small amounts of dirt or small scratches on either the blank cell or on those used for the samples may give rise to errors, which are easily overlooked, but by no means negligible. The photometer used here has therefore been provided with an absorption cell which is rigidly fixed in its place in the apparatus and which is not removed during a series of determinations. Between two determinations the cell is emptied by means of a glass tube with a finely drawn-out tip, which is used in connection with a water filter pump. Before filling a new sample into the cell it is rinsed twice with water.

Various sizes of absorption cells may be used in the photometer. The one used for ascorbic acid determinations holds 25 ml and is 4 cm long and 1 cm broad. At both ends the absorption cell is screened by diaphragms allowing only the light passing through it to reach the photocell.

In most electrophotometers the absorption cell is placed under a cover during the reading of the galvanometer to prevent false light from reaching the photocell. Besides the fact that the removal and replacing of the cover between the determinations is time-consuming, it is moreover often useful to be able to have access to the fluid in the cell during the photometry. In the present photometer the photocell has been placed at the end of an internally blackened tube 4 cm wide and about 20 cm long, at the opening end of which an iris diaphragm with a maximal opening of 2.5 cm is placed. By means of the iris diaphragm the amount of light reaching the photocell is regulated. This arrangement is sufficient to ensure that practically no false light reaches the photocell even when the absorption cell is left uncovered, except when the room is exposed to direct sunlight when light curtains will sufficiently reduce the illumination.

The photocells used are barrier layer cells of Dr. B. LANGES manufacture. They have been used in connection with two different types of galvanometers. The one is a "Cambridge pot galvanometer" which has been used in conjunction with a homemade logarithmic scale and with a working distance of 2.5 m. The other is a "Multiflex Galvanometer", type M. G. 2. The photometer described here has been in use in the laboratory for a couple of years and has proved to have many advantages over the usual

types. In making the determinations of ascorbic acid the samples have to be diluted. For this dilution either acetic acid or acetate buffers are used. In order to minimize the danger of a destruction of ascorbic acid on dilution 10 ml of a 2 % sodium cyanide solution are added to each liter of all fluids used.

The dichloroindophenole solution is made by preparing a 4 mg% solution in distilled water. This is most conveniently done by dissolving a "la Roche" tablet in 50 ml. The dissolution of the dye occurs readily even in cold water if a drop or two of 1 % sodium hydroxide is added. The solution is filtered and may be used for a few days if kept overnight in the ice box. When used it is necessary to control the strength twice a day by means of the photometer, which is done by running a blank determination in exactly the same way as in the case of an ascorbic acid determination.

If a pure ascorbic acid solution is mixed with dichloroindophenole, in the proportions described later, and the process of decolorization followed by means of the photometer it is seen that under these conditions the reaction is practically completed in  $\frac{1}{2}$  minute and absolutely so in one minute. If the sample, however, besides the ascorbic contains other substances reacting with the dye the decolorization does not stop after the lapse of a minute, but proceeds at a slower rate. The decolorization which occurs between 1 and 2 minutes after the mixing is used for an extrapolation back to zero time, thus eliminating the decolorization due to the slowly reacting substances which take place during the first minute. The validity of this correction is of course dependent on the assumption that the rate of the interfering processes is the same during the first two minutes, which is probably tolerably correct. The correction is not of course able to eliminate errors due to substances reacting with the dye at a rate comparable to that of ascorbic acid, as f. inst. thiosulphate.

In order to fix precisely the moment at which the reaction starts it is necessary that the mixing of the dye and the ascorbic acid sample is very rapid. This is ensured in the following way: 1 ml of the dye solution is placed in the absorption cell by means of a KROGH 1 ml. syringe pipette and this is immediately followed by 15 ml of the diluted sample (see later). These 15 ml are added by means of a pipette the outer tip of which has been cut so as to ensure that the pipette is emptied in 1—2 seconds. In this way the mixing of the two fluids is almost instantaneous and a definite



zero time is obtained by setting a stop watch going in the moment when the first drop falls from the pipette after the continuous delivery has stopped. The galvanometer is watched and in case the mixing is incomplete this is shown by irregular movements, in which case the fluid is stirred by means of a small glass rod. Readings of the galvanometer are taken at 1 and 2 minutes and after the last reading the remaining dye is reduced by the addition of 1 or 2 drops of a strong ascorbic acid solution (1—2 %). This leads to a rapid decolorization of the dye and after yet another half minute the galvanometer is read again, giving the light absorption due to the colour of the sample itself, which has to be subtracted from the other values in order to get the absorption due to the dye. The extrapolation is carried out by adding the difference between the two first readings (1 and 2 min.) to the reading at 1 minute. Example: Urine sample diluted with 5 % acetic acid containing cyanide and 1 % sodium acetate. Galvanometer readings: 1 min.: 186; 2 min.: 170; after decolorization: 56. Extrapolation:  $186 + (186 - 170) = 202$ . Dye remaining after the reaction due to ascorbic acid:  $202 - 56 = 146$ . This value subtracted from the dye value (230) obtained in a blank determination corresponds to the amount of dye reduced by the ascorbic acid, in this case:  $230 - 146 = 84$ .

The original dye value must be determined in a blank determination run in exactly the same manner and because the dye is not completely stable in solutions more acid than  $p_H$  4 extrapolation to zero time must be made in this case also. In order to convert the galvanometer units into ascorbic acid the apparatus must be calibrated by careful determinations on known solutions. Because of the great instability of the ascorbic acid this is no easy matter. It is best carried out by using an ascorbic acid solution, made approximately 100 mg% in cyanide containing 1 % acetic acid, and which is some days old, when it will be relatively stable. The true strength of the solution is determined by titration with iodine. Immediately before use 0.1 ml of this solution is diluted with 5 ml of the acetic acid and a determination of the dilution made by further diluting 1 ml with 20 ml of the buffer solution and using 15 ml of this for the determination. Example: Ascorbic acid mother solution determined by titration to be 77 mg%. Diluted sample thus 1.51 mg%. Buffer solution 5 % acetic acid. Readings: 1 min.: 125; 2 min.: 124; after reduction: 19. Extrapolation:  $125 + (125 - 124) - 19 = 107$ . Dye value = 234.

Reduction due to ascorbic acid =  $234 - 107 = 127$ . 127 galvanometer units thus corresponds to 1.51 mg% ascorbic acid when these dilutions are used, which means that 100 units is equal to 1.19 mg%. This factor, however, may be determined in a different way as follows: In titrations 1 mg of dichloroindophenole has been found to correspond to 0.5 mg of ascorbic acid. 1 ml of a 4 mg% solution as used in this method thus corresponds to 0.02 mg ascorbic acid, which amount must be equal to 234 galvanometer. Taking the size of the sample and the dilutions into consideration the original sample should be 2.8 mg% in order to reduce the dye completely, and 100 units should correspond to  $280/234$  or 1.20 mg% as against 1.19 mg% by direct determination. The calculation of the factor in this way is, however, only possible when the dye solution is absolutely fresh, i. e. less than 2 hours old, and if it has been prepared without heating, which may destroy some of the dye. Care must of course be taken that the dye is completely dissolved, which is easily controlled when the dye solution is filtered. The determination of the factor in this way cannot be carried out with buffer solutions more alkaline than  $p_H$  c. 3.2, where the factor found will be too small and curiously enough it has only been possible with dyes from "la Roche" and from "British Drug House", while a sample from "Merck" gave completely diverging results. If "la Roche" tablets are used the accuracy of the factor is about 5 % when calculated in this way.

The method has been adopted for the determination of ascorbic acid in different materials and as the details differ the methods are described separately.

*Blood serum:* In all methods used for the determinations in blood serum it has been the custom to deproteinize the serum. It would seem that the chance of the destruction, if any, of the ascorbic acid would be less if deproteinization could be avoided. The question of a possible interference of protein with the determination was studied on solutions of egg white to which ascorbic acid was added. The extinction of the dye proved to be the same with and without protein, and the ascorbic acid added could be determined in buffers of various acidity. The experiment was repeated with serum samples with various ascorbic acid contents and ascorbic acid added could be determined here too, when certain precautions were taken, which were necessary because the ascorbic acid added may be rapidly destroyed, as evidenced by the fact that determinations on the solution repeated at inter-

vals of a few minutes show decreasing values. The destruction which is especially rapid in serum which contains no performed ascorbic acid can be much retarded by the addition of 1 drop of 2 % sodium cyanide for each 5 ml of serum. While the experiments as to determining added ascorbic acid were successful, serums were later met with which gave negative values for the ascorbic acid content. This turned out to be caused by the fact that in contrast to what was the case with egg white, serum protein interfered with the extinction of the dye, when acetate buffers were used, while with 5 % acetic acid the values were the same with and without protein. Because of this 5 % acetic acid has been used for determinations on serum, and negative values exceeding the error of the method have not been found, while, on the other hand, zero values have repeatedly been met with either spontaneously or after the serum had been left standing for some days. By the use of a filter combination with a more narrow transparency (V.G. 9 + B.G. 7) it is possible to work with a buffer solution of  $p_H$  3.9 and yet obtain practically correct results, which are, however, somewhat lower (0.10—0.15 mg%) than those obtained with 5 % acetic acid. The use of this buffer may have some advantage as the dye is more stable in this than in the unbuffered acetic acid, but the results with the latter are probably the most correct. The determination is consequently carried out in the following way: Blood is drawn, and after the addition of 1 drop of 2 % sodium cyanide for each 5 ml it is centrifuged rapidly before coagulation sets in. After a few minutes centrifugation, the coagulum now formed is loosened from the wall of the tube and the centrifugation renewed, till serum is obtainable. 1 ml. of serum is mixed with 20 ml of 5 % acetic acid containing cyanide and the determination is carried out on 15 ml of this mixture as described above. The mean error of this method has been determined on a series of 50 sets of routine double deter-

terminations<sup>1</sup> according to the formula  $m.e. = \sqrt{\frac{\sum d^2}{2n}}$ , where  $d$  is the difference between the individual double determinations and  $n$  the number of sets. The mean error on the single determination was 0.023 mg%.

That the substance determined in this way is really ascorbic acid is indicated by the fact that the value found in the blood of a

<sup>1</sup> My best thanks are due to Miss A.-L. LINDBERG for performing these analyses and for other help in working out the method.

certain individual falls rapidly when ascorbic acid is excluded from the diet and that the values found fluctuate closely with the intake of ascorbic acid, so closely in fact that the increase due to the ingestion of 25–50 mg ascorbic acid may be demonstrated. As described here the method calls for a fairly large blood sample, but by using a smaller absorption cell it is possible to carry out the determination on much smaller samples. Using a homemade absorption cell 4.7 cm long, 3.5 mm broad and 15 mm high. F. LUNDQUIST working in this laboratory has been able to reduce the size of the serum sample to 0.1 ml which is diluted with 1 ml of acetic acid and added to 1 ml of a diluted dye solution (0.1 mg%) in the absorption cell. The determinations made with this micro-method were almost as accurate as those made with the macro-method, though no actual determination of the mean error has been made.

*Urine.* The determination of the ascorbic acid content of urines encounters just as great difficulties as is the case with serum, because the amount of interfering substances is large, and moreover it seems that some of the interfering substances react with dichloroindophenole with a velocity which at least at some levels of  $p_H$  is almost the same as that with which ascorbic acid reacts with the dye. If a sample of fresh urine is allowed to react with the dye in the photometer, the decolorization curve is at the beginning very similar to that of an ascorbic acid reaction, but the reaction is not completed after the lapse of 1 minute, but proceeds, just as in the case of serum, at a slower but more constant rate, evidently due to other reducing substances. If samples of the same urine are analysed at intervals, the height of the rapid part of the curve is seen to decrease rapidly, while the slower part remains comparatively unchanged, indicating that the most labile substance (ascorbic acid) is oxidized by the atmospheric oxygen, while the less reactive ones are left unchanged. In the course of a day or two the rapid part of the curve may disappear completely but if ascorbic acid, calculated to correspond in amount to that which has disappeared, is added, the curve is restored to practically the old form (fig. 2). This experiment may be carried out with the same result using various buffer solutions for the dilution of the sample. If, however, the height of the first part of the curve is compared at various  $p_H$  levels, it is found that the height decreases as the buffer gets more alkaline, as is the case too with curves from ascorbic acid, but the decrease is much larger with

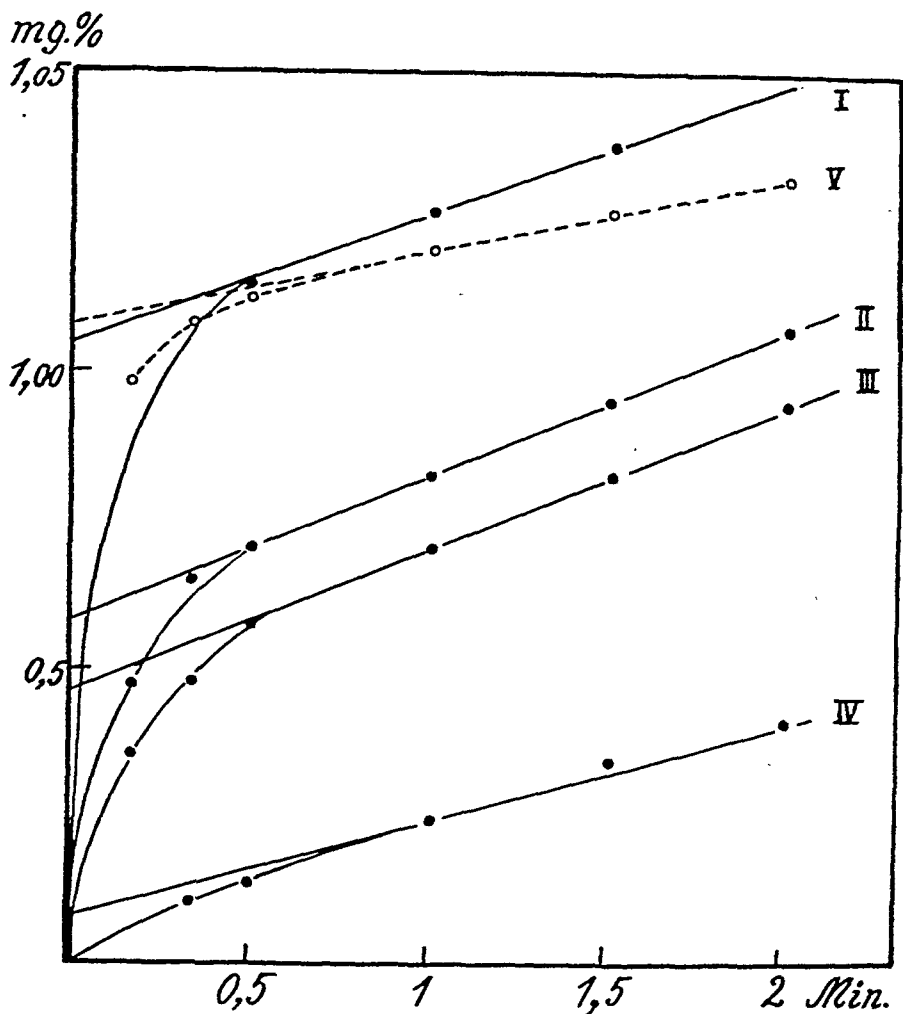


Fig. 2. Reduction curves for samples from the same urine at intervals. I fresh urine; II after 80 minutes; III after 140 min.; IV after 23 hours; V after 23 hours and with the addition of 1 mg% ascorbic acid.

urine, which must indicate that the whole height of that part of the curve cannot be due to ascorbic acid alone, but that other substances must interfere. If buffer solutions more alkaline than about  $p_H$  4.5 are used the results, however, agree with those on ordinary ascorbic solutions, and because of this it is regarded as most likely that results obtained with the more alkaline buffers correspond best with the true ascorbic acid content of the urines. The abnormal behaviour in the more acid buffers is perhaps due to thiosulphate, as it can be demonstrated that this substance in pure acetic acid reacts with dichloroindophenole at a rate, which is about half of that at which ascorbic acid reacts, while

the rate decreases to a very low value at  $p_H$  4.5. The effect of an eventual content of thiosulphate in the urine will therefore be eliminated when the determination is made at this  $p_H$ . It must be emphasized, however, that neither with urine nor with serum does the mere fact that added ascorbic acid may be determined offer any guarantee that the substance determined in the native fluids is ascorbic acid. As it is not possible to use the same indirect proofs (f. inst. fluctuations with small variations in intake) as in the case of serum, all that can be said with regard to urine is that the substance determined by the method reacts with dichloroindophenole at the same rate as ascorbic acid does. The method proposed for urine is as follows: 1 ml of urine is diluted with 20 ml cyanide containing 1 % acetic acid with 1 % sodium acetate. 15 ml of this mixture is used for the determination as above.

*Milk.* The determination of the ascorbic acid of milk calls for a precipitation of the proteins + the fat in order to obtain a fluid sufficiently clear to allow photometry. The precipitation has been performed by adding 3 ml of a fresh 2 % solution of metaphosphoric acid to 1 ml of milk, which has been centrifuged and filtered, and subsequently diluting with 25 ml of a buffer containing 5 % acetic acid and 0.2 % sodium acetate + cyanide. The solution is filtered through a filter (f. inst. SCHLEICHER und SCHÜLL 589<sub>2</sub>) which clears the fluid sufficiently to allow photometry and which does not give off reducing substances in amounts large enough to invalidate the determination. 15 ml. of the filtrate are used for the photometry as above. The mean error on the single determination calculated from 25 sets of routine double determinations has been 0.025 mg%. The factor for the conversion of galvanometer units into ascorbic acid may as in the case of serum be calculated from the dye value; because of the different dilution the factor is here equal to 3.86/dye value.

*Other materials.* The method may of course be used on all materials capable of giving a fluid clear enough to allow photometry. It has been used in the laboratory for a number of determinations on plant material, which was extracted with cyanide containing 5 % acetic acid. This solution is not acid enough to hinder the action of the oxydases in cold extraction, so that the extraction has been made by addition of the material to the boiling acid, whereby the oxydases are immediately destroyed. Generally the fluid does not call for a filtration, but larger particles may

be avoided by filling the 1 ml pipette through a small glass tube provided with a plug of cotton. On a series of determinations on the contents of extracts of hips, the mean error on the single determination calculated from 25 sets of double determinations has been 0.011 mg%.

### Summary.

Various redox-indicators have been tried in determinations of ascorbic acid.

Dichloroindophenole seems to be best suited for electrophotometric determinations.

A simple but improved electrophotometer is described.

Methods for the determination of ascorbic acid in various biological fluids are described. In the serum the determination may be carried out without protein precipitation.

In urine substances are present, which contrary to the general conception interfere most in the more acid buffers.

The best results are obtained with buffers of a  $p_H$  around 4.5.

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## The Antinarcotic Effect of Estrone on Alcohol Intoxication.

By

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The resistance of humans and animals to the action of narcotics and toxic substances is subject to great individual variations. Several authors have searched for the cause in the endocrinal field. Thus the toxic effect and the metabolism of ethyl alcohol have been investigated in relation to hormonal influence.

*Insulin* is said to accelerate the alcohol metabolism in rabbit (SUPNIEWSKY 1926, KANITZ 1936, JUNGE 1937), in dog (WIDMARK 1935, CLARK, MORRISSEY, FAZEKAS and WELCH 1941), as well as in man (NEWMAN and CUTTING 1935, GOLDFARB, BOWMAN and PARKER 1939, MEYER 1939). Contrary to this MIRSKY and NELSON (1939) proved no action of insulin on blood alcohol in pancreatectomised dogs, neither did FLEMING and REYNOLDS (1935) nor BLOTNER (1939) find any effect on oxidation rate in man. — With regard to the absorption of alcohol from the intestinal tract insulin had an inhibitory effect (SIEGMUND and FLOHR 1937, BÖHMER 1938). — Its effect on the degree of intoxication was primarily "psychic", and no effect whatsoever on the neuro-muscular coordination (JUNGE 1937).

*Thyroxin* produced no effect on the combustion rate of alcohol, either on experimental animals or man (WIDMARK 1935, LANG and SCHLICK 1936, BENEDICT and MEZEY 1937). On the other hand, by determining *in vitro* the  $O_2$ -metabolism, DONTCHEFF (1939) found that thyroxin reduced the alcohol oxidation rate, probably by way of an enzymatic reaction.



*Adrenaline* and *pituitrin* have no effect on the alcohol combustion (WIDMARK 1935, FLEMING and REYNOLDS 1935); the alteration of the blood alcohol curve by these hormones being due to their inhibitory effect on the absorption of ingested alcohol (SIEGMUND 1937, BLOTNER 1939).

With regard to *sex* and *sex hormones* ABDERHALDEN and WERTHEIMER (1927) indicated a sex difference in alcohol tolerance, female mice being more resistant to chronic alcohol poisoning than males. KASK (1929) claims that castrated rabbits were affected by smaller doses of alcohol than normal animals. STÖRTEBECKER (1937 a and b, 1939) proved estrogens and androgens to have an acute antinarcotic and antitoxic effect on mice and rabbits against different drugs, *i. a.* ethyl alcohol. Thus castration lowered the resistance to alcohol, and treatment with estrogens, on the other hand, increased it.

Studying alcohol addiction in rats, KLOTZ (1937) found ovariectomy to induce an increased sensitivity to alcohol. AGDUHR (1938) stated that normal sexual functions intensified the resisting power of the experimental animals to alcohol intoxication, mated animals being compared with animals kept isolated. Finally IJIRI (1939), using the Warburg apparatus found that the  $O_2$ -metabolism of brain substance from castrated animals was decreased by a 0.5 % alcoholic solution, normal brain substance not being influenced.

From the above it is obvious that certain hormones may influence the alcohol metabolism and the degree of intoxication, but for the sex hormones the mode of action is not perfectly clear.

In the present work we intend to study

- 1) whether estrone produces its antinarcotic effect (STÖRTEBECKER 1937 b) by means of a general change in alcohol metabolism or whether it is only local, and
- 2) whether the existing variations in the individual resistance to alcohol are due to the antinarcotic effect of estrone.

### Methods.

The experimental animals were castrated female rabbits, used 1 to 8 months after ovariectomy, and weighed 2350 to 3400 g. Criteria of alcohol intoxication were those established by GOLDBERG and STÖRTEBECKER (1941) and were referred to the prevailing blood alcohol level.

On one and the same animal at least two experiments were performed, the first with alcohol, the second with alcohol and estrone. The ethyl alcohol was injected intravenously in a dose of 2.4 to 2.8 g alc. abs. per kg body weight, diluted to 33 % by volume, infusion rate 1 ml per min.

*Estrone treatment:* Crystalline estrone,<sup>1</sup> 8 to 15 mg = 80,000 to 150,000 int. units, was administered simultaneously with the alcohol injection, the estrone being dispersed in the first portion of the alcohol solution. The estrone brought about none of the symptoms, characteristic of alcohol intoxication, nor did it interfere with the alcohol analysis.

*The alcohol analysis* was carried out according to WIDMARK's micro-method (1932). The constants  $\beta$  and  $r$  (WIDMARK 1932) were calculated after the method of least squares, likewise the standard errors of  $\beta$  and  $c_0$  (BERNHARD and GOLDBERG 1935). The antinarcotic effect was determined as the difference in blood alcohol concentration for one and the same criterion before and after estrone treatment (cp p. 293 and table 1).

## Results.

Alcohol was administered to castrated female rabbits before and after estrone treatment in altogether 9 series (18 experiments), all of which were performed under uniform conditions: each series on one and the same animal at constant dosage and body weight ( $\pm 100$  g).

*Symptoms.* After finished intravenous injection of 2.4 to 2.8 g alc. abs. per kg the castrated animals showed the following reflex state: righting reflexes, spontaneous activity and normal sitting posture (chin sign) were all absent, and the typical alcoholic postural nystagmus was present. — During recovery from the intoxication, the reflexes reappeared in the order to be seen in fig. 1, the postural nystagmus generally being the last criterion of intoxication to disappear.

The estrone-treated castrated animals recovered from the alcohol intoxication at an earlier stage than before treatment. Furthermore the sequence of the reappearing reflexes changed during recovery: the spontaneous activity generally beginning before the hind limb righting reflex became present, and the animals resuming their normal sitting posture (chin sign +) almost simultaneously with the appearance of the righting reflexes, a striking difference from the untreated animals (cp. fig. 1). These observations confirm the experiments of STÖRTEBECKER (1937 a, b, 1939)

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<sup>1</sup> We wish to express our best thanks to ERIK JACOBSEN, M. D., A/S Medicinalco, Copenhagen, for so kindly supplying us with estrone.

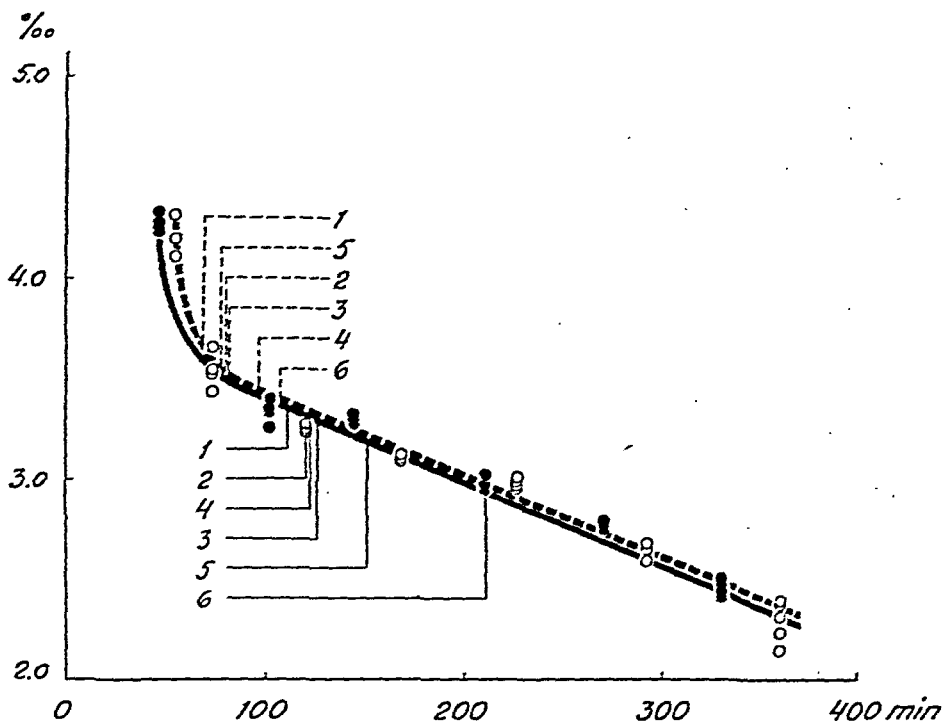


Fig. 1.

*Criteria of Alcohol Intoxication Referred to Blood Alcohol, before and after Estrone Treatment.*

- ■ ● Castrate. Rabbit 5. 3,190 g. 2.8 g alcohol per kg  
 $\beta = 0.0041\text{ ‰/s}$   $c_0 = 3.82\text{ ‰}$   $r = 0.73$
- ■ ○ Castrate + Estrone. Rabbit 5. 3,060 g. 2.8 g alcohol per kg + crist.  
 estrone 150,000 int. units  
 $\beta = 0.0041\text{ ‰/s}$   $c_0 = 3.84\text{ ‰}$   $r = 0.73$
- Criteria present: 1 = Righting reflexes: Head 4 = Nystagmus  
 2 = " " Forelimbs 5 = Spontaneous activity  
 3 = " " Hindlimbs 6 = Normal Sitting Posture

with estrone treatment of castrated and normal rabbits and white mice.

*Blood alcohol curve.* The diffusion phase lasted 30 to 80 min. and generally had an exponential course. — The following post-absorptive combustion phase was rectilinear in all cases, the curves agreeing completely with those found in normal animals (NEWMAN and LEHMAN 1937, GOLDBERG and STÖRTEBECKER 1941).

The oxidation rate  $\beta$  and the distribution factor  $r$  (WIDMARK 1932) were determined in 6 series:

$$\beta \begin{cases} \text{before estrone treatment: } 0.0045\text{ ‰/s} \pm 0.0003 \\ \text{after " " : } 0.0043\text{ ‰/s} \pm 0.0003 \end{cases}$$

$$r \begin{cases} \text{before estrone treatment: } 0.88 \pm 0.04 \\ \text{after " " : } 0.86 \pm 0.04 \end{cases}$$

Thus estrone treatment of castrated animals had no influence on  $\beta$  nor on  $r$ , and the values found agreed completely with those established for normal animals:  $\beta$  being  $0.0048 \text{ } \text{‰} \pm 0.0002$ , and  $r$  being  $0.87 \pm 0.02$  (GOLDBERG and STÖRTEBECKER 1941).

*Symptoms related to blood alcohol.* After estrone treatment the reflex data occurred sooner. With the blood alcohol curve being unchanged, an earlier occurrence of the reflex data must correspond to a higher blood alcohol level (see fig. 1, illustrating the relation between symptoms and blood alcohol).

The size of this difference in blood alcohol concentration for one and the same reflex criterion has been used as a measure for the antinarcotic effect of estrone. The difference was greatest for spontaneous activity and normal sitting posture (chin sign), considerable for righting reflexes and suggested for postural nystagmus (table 1).

Table 1.

*Criteria of Alcohol Intoxication Referred to Blood Alcohol, in Castrated Animals before and after Estrone Treatment.*

Criteria	Blood Alcohol Concentration		Antinarcotic Effect Difference in Blood Alcohol $\text{‰}$
	before Estrone $\text{‰}$	after Estrone $\text{‰}$	
Righting Reflexes			
Head . . . . .	3.03	3.30	$0.27 + 0.07$
Forelimbs . . . . .	2.97	3.23	$0.26 + 0.07$
Hindlimbs . . . . .	2.82	3.09	$0.27 + 0.05$
Spontaneous Activity .	2.68	3.12	$0.44 + 0.08$
Normal Sitting Posture (Chin Sign) . . . . .	2.63	3.03	$0.40 + 0.11$
Nystagmus . . . . .	2.56	2.72	$0.16 + 0.10$

The values are means of 8 series of experiments.

Experiments have shown, that the difference in blood alcohol, viz. the antinarcotic effect, becomes greater at a higher alcohol dosage, 2.8 to 3.25 g per kg body weight, why this amount ought to be used for further work on this theme.

In all the estrone experiment the animals showed a more rapid recovery than those untreated. In some cases, however, the animals relapsed into a higher degree of intoxication after 3 to 5 hours, the reflexes already present disappearing. This change in

the reflex state was not accompanied by a similar change in the blood alcohol curve, which was still rectilinear, neither did it appear in untreated nor in normal animals. Their relapsing in these cases must be ascribed to the antinarcotic effect of estrone being transient. STÖRTEBECKER's (1937 a) observations with ether are in favour of this assumption; he found an antinarcotic effect of estrone, provided the hormone being administered together with the ether anesthesia; but estrone given the day before produced no visible effect. This transient effect of estrone is due to the estrone being inactivated rapidly in the organism. Thus *i. a.* DINGEMANSE and LAQUEUR (1937) could only find traces of estrone in the body 6 hours after administration of even large quantities (25000 int. units), dissolved in water or in oil. The transient effect seems to be common to the hormones of the gonads, androsterone and testosterone also disappearing rapidly from the organism.

The earlier described nystagmic opistotono-rotatoric syndrome (GOLDBERG and STÖRTEBECKER 1941), visible at heavy alcohol dosage ( $> 3.0$  g per kg), was also found in castrated animals as well as after estrone treatment, under the same conditions as in normal ones. This syndrome could be inhibited, provided the nystagmus was prevented by keeping the head of the animal fixed in a normal upright position. DANDY and KUNKEL (1939) have found a similar whirling in dogs and cats after section of one or both VIIIth cranial nerves.

### Discussion.

The antinarcotic effect of estrone on alcohol intoxication (STÖRTEBECKER 1937 b) has been confirmed. Our present observations show that this effect cannot be explained by a lowering of the blood alcohol level, as estrone treatment produced no change of the blood alcohol curve. The effect of estrone on alcohol intoxication must thus be looked for in the central nervous system.

The altered relation between the reflex symptoms and the blood alcohol concentration means a changed threshold of the central nervous system to alcohol after estrone treatment; the reflex data occurred sooner, corresponding to a higher blood alcohol concentration. Analogous to this the narcotic concentration for ether in the blood was 82 mg% in castrated rabbits, and it increased to 126 mg% after estrone treatment (STÖRTEBECKER 1937 a).

Spontaneous activity and normal sitting posture were less affected by alcohol than other reflexes after estrone treatment, and this different affection of the symptoms indicates a certain localization of the estrone effect to higher nervous functions. JUNGE (1937) has observed a similar phenomenon with insulin treatment, which caused a "psychic" awakening effect, consisting in spontaneous activity and excitation, though the neuro-muscular incoordination (ataxia etc.) remained.

*Resistance.* Concerning the individual variations in resistance to alcohol, our observations show that estrone treatment diminishes the degree of alcohol intoxication in castrated as well as in normal animals. This difference in resistance to alcohol before and after estrone treatment is especially marked in the "behaviour" (spontaneous activity and normal sitting posture). Further the normal animals have a greater resistance to alcohol than those castrated (STÖRTEBECKER 1937 b). The antinarcotic effect of estrone on alcohol intoxication involves a change in the relation between reflex symptoms and the blood alcohol level, the general alcohol metabolism not being influenced, indicating that estrone alters the excitatory state of the central nervous system. These facts enable us to conclude that resistance to alcohol intoxication is related to the hormonal state, and that the amount of estrogens in the organism is an important factor in this respect.

### Summary.

The effect of estrone treatment on ethyl alcohol intoxication in castrated rabbits has been studied; the degree of intoxication being established by means of reflex criteria and referred to the blood alcohol level.

1. Estrone treatment diminishes the degree of alcohol intoxication, STÖRTEBECKER'S (1937 b) observation being confirmed.

2. After estrone treatment the animals only attain the same degree of intoxication at a higher blood alcohol level. The difference in blood alcohol concentration has been used as a measure for the antinarcotic effect of estrone.

3. The general alcohol metabolism is not affected by estrone, the oxidation rate  $\beta$  and the distribution factor  $r$  being unaltered. The effect of estrone on alcohol intoxication must thus be searched for elsewhere, *e. g.* in the central nervous system.

4. The varying resistance to alcohol depends *i. a.* on the hormonal state of the individual, and the amount of estrogens in the organism seems to be an important cause.

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## On the Water Transport through the Gills of Bivalves.

By

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The large majority of bivalves feed, as is well known, on particulate material, micro organisms or detritus, suspended in the water respired, and retained during the passage of this water through the gills. In studies of the nutrition biology of lamelli-branchs the quantitative determination of this water transport is therefore important.

Several attempts have been made to carry out such determinations, but only in a few cases has a technique been employed which could be expected to give even approximately reliable results.

Both direct and indirect determinations have been attempted. GALTSOFF (1928 a and b), working directly, introduced a glass tube into the expiration siphon leading the water to a measuring cylinder in which the quantity of water transported could be read off, care being taken to avoid any pressure difference between external and internal gill surfaces, or the transport was judged from measurements of the rate of movement of a carmine suspension in the tube connected with the siphon. The objections against these experimental procedures are obvious. The introduction of a tube in the siphon and a glass rod between the valves to prevent their being closed interferes so seriously with the normal conditions of the animal that the values found will probably be too low. Moreover it was shown by HOPKINS (1933) that the rate of water transport depends not only on the



ciliary movements on the transporting surfaces, especially those of the gills, but is also regulated by the degree to which the shells are opened. This, like the ciliary movements, is influenced by temperature, but the optimum is different from that of the cilia. Any regulation of the position of the shells is of course excluded when a glass rod is put in between them. The applicability of the method is restricted to forms possessing, like GALTISOFF's experimental animals, a suitable anal siphon.

The principle in the indirect methods most often attempted has been to calculate the quantity of water transported through the gills from the removal of particulate material of suitable dimensions and density. VIALLANES (1892), DODGSON (1928), DAMAS (1935) and FOX, SVERDRUP and CUNNINGHAM (1937) have utilized this principle, but FOX is the sole author who worked it up into a method.

Fox placed his experimental animals (*Mytilus californiensis*) in suspensions of  $\text{CaCO}_3$  and determined the reduction in the Ca content, brought about by the mussels, by Ca-analyses at suitable intervals. The amount of water filtered is calculated from the formula

$$p_t = p_0 \cdot e^{-\left(\frac{n \cdot m}{M} - a\right) \cdot t}$$

$p_0$  is the Ca content at the start of the experiment and  $p_t$  the content after  $t$  hours,  $n$  is the number of mussels used.  $M$  is the number of liters of suspension used in the experiment, and  $m$  the quantity in liters transported through the gills per hour and per animal.  $a$  finally is a correction allowing for the sedimentation taking place independently of the mussels. This sedimentation was measured in control experiments.

The formula can only give absolute values provided the calcium is quantitatively retained by the gills. The results seem to show that this was really the case, since  $\log p$  was found to be a linear function of  $t$  for values of  $p$  at all the concentrations examined, ranging from 1.24 to 0.47 g Ca. per liter. This will be the case only if the mussels retain all the Ca passing through the gills or if a constant percentage is retained, independently of the concentration. This latter possibility appears improbable. It is therefore to be assumed that the Ca is practically completely removed from the suspension in the transport through the gills.

The results of Fox' experiments are shown in table I.

Table I.

Number of mussels used	Number of experiments	Weight of mussels in g.		Transport of water in 1 per hour per mussel	
		Range	Average	Range	Average
6	4	337—515	431	1.8—18.1	6.4
24	6	75—166	93	2.2— 2.9	2.6
26	4	34— 40	37	0.5— 2.1	1.4

The method of Fox and collaborators is a great improvement on those formerly employed, but the large quantities of calcium carbonate necessary (ca 3 g/l) are a drawback. The dry matter of the suspension is far above the normal, and small bivalves or pelagic larvæ will in any case be adversely affected by the enormous quantities of material in suspension and give much too low values for the transport. Only fairly large individuals have been used therefore, (from 34 g upwards). The chemical Ca determinations necessary make the method rather time consuming.

The suspended material to be used in such experiments must fulfill the following conditions.

It must form a stable suspension of grains of uniform and suitable size which can be retained completely by the gills. It must be nonpoisonous and not produce irritation (e. g. by changing the  $p_H$  of the water). It must be easy to determine quantitatively and easy to procure. The suspension used by Fox et. al. corresponds only in part to these conditions.

The search for such a material led to the choice of the blue-green alga *Synechococcus*. This alga was cultivated in sea water from the locality where the experimental animals (*Mytilus edulis*) were taken. Soil extract and nutritive salts were added according to the directions of SCHREIBER. The  $p_H$  was kept constant by the frequent addition of dilute HCl. In vigorous cultures showing a maximal growth no sedimentation of algæ takes place, and the dry matter of the water is much closer to the normal values than is the case using Fox' method.

In the experiments about 1 g mussels were placed in 200 ml *Synechococcus* culture. I tried in the first experiments to determine the concentration of cells by counting in a suitable chamber under the microscope. But the number was reduced so quickly that a large number of

counts became necessary to obtain a sufficient accuracy. The specific gravity of the algæ is so close to that of the sea water that a concentration of the cells by centrifugation is impossible. It was preferred therefore to make a photometric determination of concentration by measuring the absorption of light in a definite thickness of the suspension. The electrophotometer according to REHBERG was used (*Acta physiol. Scand.* 1943). Determinations were made on 20 ml samples, and filter RG 1 was used.

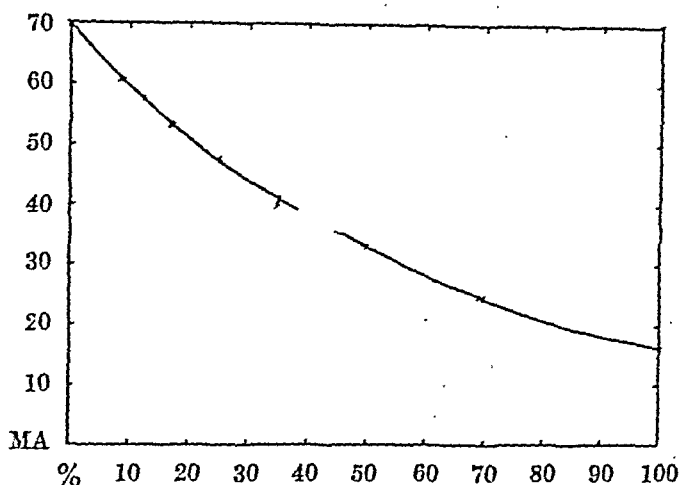


Fig. 1.

The light absorption caused by dilution of various suspensions of the algæ is shown in fig. 1. The most concentrated culture is put at 100 (corresponding in the photometer used to a current of 16.2 MA, when 0 % (pure water) was equivalent to 70 MA). The curve was first drawn on the basis of dilutions to  $\frac{1}{2}$ ,  $\frac{1}{4}$  and  $\frac{1}{8}$  of the strongest culture and the readings corresponding to these dilutions. Thereupon another (weaker) culture was diluted in the same way. The undiluted weaker culture was measured on the curve for the first culture and found to correspond to 69.6 %. Accordingly the rest of the dilutions should correspond to 34.8, 17.4 and 8.7 % of the strongest culture and they are found to fit the curve. A third culture treated in the same way likewise fitted the curve. It follows that when only fresh and vigorous (distinctly blue-green) cultures are used it is possible to express the concentration of any culture and its dilutions in per cent of the standard culture.

We must assume that the number of cells removed by the mussels during an experiment is at any time proportional to the number present, and the removal can therefore be expressed as a monomolecular reaction according to the formula

$$\text{conc}_t = \text{conc}_0 \cdot e^{-\frac{m}{M} \cdot t} \quad \text{or} \quad m = \frac{(\log \text{conc}_0 - \log \text{conc}_t) \cdot M}{\log e \cdot t}$$

in which  $M$  is the quantity of water in ml used for the experiment and  $m$  the quantity transported per min. while  $conc_0$  and  $conc_1$  are taken from the curve in accordance with the ampèremeter readings.

$m$  will represent the absolute flow of water, only if all cells passing the gills are kept back. It is reasonable to assume that at least a very large majority of the *Synechococcus* cells which are only 2—3 mikron long are so retained by the gills of *Mytilus*. ZOBELL and LANDON (1937) state that 99.9 % of added bacteria are retained by *Mytilus californiensis*, but GALTISOFF found that only 20—50 % of the bacteria in sea water were retained by *Ostrea virginica*. Large variations in the retaining power of the gills of different bivalves seem to exist therefore.

The concentration of the algae can probably be determined also by colorimetry. Fresh vigorous cultures show a very strong blue-green colour. The chief advantage of the "alga method" are the rapidity with which it can be carried out, its lack of interference with the normal life of the experimental animals and its consequent reliability. It will be possible to use it on all kinds of plancton filtering animals. It is a drawback that fairly large quantities of fresh cultures are required for experiments on a larger scale.

Only a small number of determinations have been carried out so far by means of this method.

I give here only the result of 3 sets of experiments on the same sample of small *Mytilus* (5 specimens weighing 1 g in all). The concentration of the algae is determined with intervals of 1—2 hours.

Table II.

	I	II	III
Temperature	11° C	22° C	22° C
ml water	139	86	186
per hour	140	150	136
	45		

The duration of the experiments was in I 2.5, in II 2.7 and in III 2.6 hours.

The large variation is no doubt due to the circumstance that the mussels have not been active all the time during the experiments. The highest figures must be nearest the true transport which is probably even a little higher.

The experiments show that the transport of water is proportionally much larger in the young individuals than in the adults. FOX gives the transport as 10 ml for the largest and 40 ml for the smallest individuals.

I have found it possible also to make a comparison with pelagic larvæ of bivalves, having found in the literature as set of figures from which the necessary calculations can be made. It was published by BRUCE, KNIGHT and PARKE (1940). BRUCE used the material to calculate the number of micro organisms taken up by veliger larvæ of the oyster in a culture of flagellates of 3—5 mikron. The water was kept in steady motion so that the distribution both of larvæ and of flagellates remained uniform. The results are seen from the table III.

Table III.

D a t e	Number of micro organisms per cubic millimeter	
	Experiment	Control
8. aug. . . . .	76	78
9. ' . . . .	56	75
10. ' . . . .	52	76
11. ' . . . .	32	65
12. ' . . . .	15	72

There were 10,000 larvæ with an average length of 200 mikron in 16 l of the flagellate suspension. The temperature was 20—22° C. The average water transport for the whole of the experimental period expressed in cubic millimeter per larva per hour is

$$m = \frac{(\log 76 - \log 15) \cdot 1.6 \cdot 10^7}{0.434 \cdot 96} = c \ 27.$$

This figure again can be absolute only if we assume that the flagellates are retained quantitatively. This is almost certainly the case by reason of their comparatively large size.

When the water transport is calculated from the fall in concentration from one day to the next we find rather variable figures (table IV).

These variations are no doubt due to errors in the counts as exemplified by the variations in the control figures. A rise in the water transport during the experimental period, as apparently indicated by the figures, is probable, because of the growth of the larvæ. The flagellates used make excellent food for oyster larvæ.

Table IV.

	Water transport in mm <sup>3</sup> per larva per hour
8.— 9. aug. . . . .	20
9.—10. ' . . . .	5
10.—11. ' . . . .	32
11.—12. ' . . . .	50

Oyster larvæ of 200 mikrons length have approximately the same volume as a sphere of 180 mikrons diameter. The volume is then about 0.003 mm<sup>3</sup>. Supposing the specific gravity to be 1.33, the weight per larva is 0.004 mg or 250,000 larvæ will make up 1 g. This means that 1 g larvæ will transport about 6.7 l water per hour, which is about 35 times the quantity transported by young mussels and up to several hundred times the quantity transported by adults. It is possible, however, that the figures found by Fox are too small, as the large quantity of lime used in his experiments must mean a severe strain upon the transporting organs, the gills especially.

But even if the true transport is larger than found by Fox, the order of magnitude will probably remain the same, and the large differences between veliger larvæ, young bottom stages and adults will remain. The water transport per g weight for the different weight groups is given in table V.

Table V.

Weight of the mussel in g.	Transport of water in l per g and hour
0.000 004	6.7
0.2	0.19
37	0.04
93	0.03
431	0.01

In this investigation I have received valuable help from dr. A. KROGH (advice and critical revision of the paper) and from dr. P. BRANDT REHBERG (especially regarding the use of the electrophotometer). I wish to express my most sincere thanks for this aid.

### Summary.

The water transport through the gills of young mussels (*Mytilus edulis*) has been calculated by means of photometric determinations of the percentage amount removed in unit time of the unicellular alga *Synechococcus* from a known quantity of a suspension of the algæ.

Having found in the literature a set of figures, from which the necessary calculations could be made, a comparison with pelagic larvæ (oyster) has been possible. It has been found that 1 g larvæ will transport 6.7 l water per hour, young bottom stages 0.19 l and adults 0.01—0.04 l water per hour and per g.

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## A Centrifugation Method of Ultrafiltration Using Cellophane Tubes.

By

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Received 29 January 1943.

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Ultrafiltration is a useful method in the study of many biochemical problems the use of which, however, is restricted by the fact that it is a slow and cumbersome process calling for complicated apparatus, if the volumes dealt with exceed about 20 ml and is very ineffective with smaller volumes. The introduction of centrifugation as a means of producing the necessary pressure (DE WAARD, 1918) was a great advantage, but the method has not been used to any extent in research work. One reason for this is probably the difficulty in preparing the filtering tubes. These have usually been made by preparing porous clay tubes with various membrane forming materials. TOTH (1927) tried tubes prepared both with collodion and with viscose cellophane and found both useful. BRINKMAN and STEINTOORN (1936) worked with tubes the inside of which were covered with a layer of sodium silicates (water glass) and HAAHTI with collodion impregnated tubes. The use of water glass coated tubes may be useful in some cases, especially as these tubes are very easy to prepare. The use is, however, restricted by the alkaline reaction of the coating which in many cases makes the use impossible. One disadvantage of the tubes impregnated with collodion is that they must necessarily be kept in a moist state in order to preserve their permeability. This involves that the first part of the ultrafiltrate formed is diluted by the water present in the pores of the clay tube which, when the volume of the filtrate is small, may introduce not inconsiderable errors. A great advan-



tage of the collodion tubes is their very large filtering capacity. The preparation of cellophane coated tubes by the viscose process is too difficult to be of much value in ordinary laboratory work. The use of cellophane membranes would, however, as in the case of colloid osmotic pressure, be a great advantage, as such membranes can be kept dry till the moment when they have to be used, so that no dilution of the ultrafiltrate needs take place. It was consequently tried whether cellophane membranes prepared according to the cellulose acetate method could be used instead of the viscose cellophane, but this proved not to be the case as membranes of this type were practically impermeable to water. Thanks to the kind help of the director of the cellophane factory "Kapcello", civil engineer H. HAVLIK, who has spared no trouble in this matter, it became possible to try tubes of viscose cellophane and a technique for using factory prepared membranes was worked out. This technique has proved very valuable in the work of this and other laboratories and has been used in a number of studies of different character.

The membranes have been used as pure cellophane tubes, not as cellophane prepared clay tubes. They are furnished in the moist state by the factory<sup>1</sup> as cylindrical tubes 9—10 cm. long and with a diameter of 17 mm. (Fig. 1, a), similar to the cellophane capsules used to put over the glass stoppers of medicine and perfume bottles. A smaller size of the tubes having only a diameter of 13 mm. may also be used. In the laboratory they are kept in distilled water to which has been added some drops of tricresole. When a tube is wanted for use it is placed in running tap water for a couple of hours or, what is more practical, the tubes which it is expected to use the next day are washed overnight. After that they are rinsed a few times with distilled water. The drying is carried out on a tinned iron cone (Fig. 1, b), the surface of which is moistened with a drop of paraffine oil, before the wet cellophane tube is drawn over it. The upper end of the cone has a groove of 1 mm. depth which serves to prevent a lengthwise contraction of the tube during the drying process. As further security against this a rubber band is placed around the tube at the upper end (Fig. 1, c). The drying may be carried out either at room temperature or in a heating box at a temperature of 60°. The progress of the drying may be followed visually; when the drying is complete the opacity disappears

<sup>1</sup> "Kapcello", Mosevej, Copenhagen, which will send specimens on request.

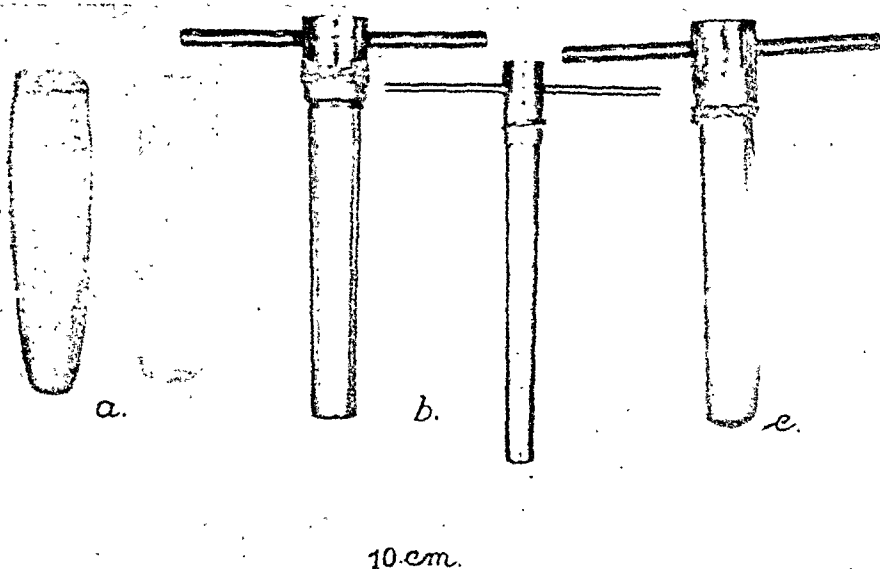


Fig. 1.:

a. wet cellophane tubes. b. drying cones. c. cone with tube ready for drying.

and the tube takes on a clear cellophanelike appearance. If it is wanted to dry a tube rapidly this may be done by placing it for some minutes in alcohol, drying it by clapping with filter paper, rinsing it with ethyl ether and then drying it in the heating box. The dry tube is loosened from the cone by cautious turning movements of the hand. If it sticks too tightly to the cone it may be loosened by passing it rapidly once or twice through a steam jet, or by holding it over some steaming water.

The tube is now very similar to a dry cellophane centrifuge tube (Fig. 2, a), and it may be kept in this state for a long time, if placed in a closed container with some moist filter paper. For use it is placed in a tubeformed slightly conical basket made of metal wire netting of such dimensions that it will just allow the dry cellophane tube to slip down into it, when it is pressed down by means of a blunt rod (Fig. 2, g). Our baskets have been made of nickel wire netting, having about 60 meshes pr. sq. cm., while the bottom and the seam, which is necessary on one side of the basket have been made from stainless steel. The finished basket has been tinned.<sup>1</sup> During centrifugation the cellophane tube is pressed against the meshes of the netting and this is usually

<sup>1</sup> The metal parts used in the method may be had from the workshop of the Zoophysiological Laboratory, Copenhagen.

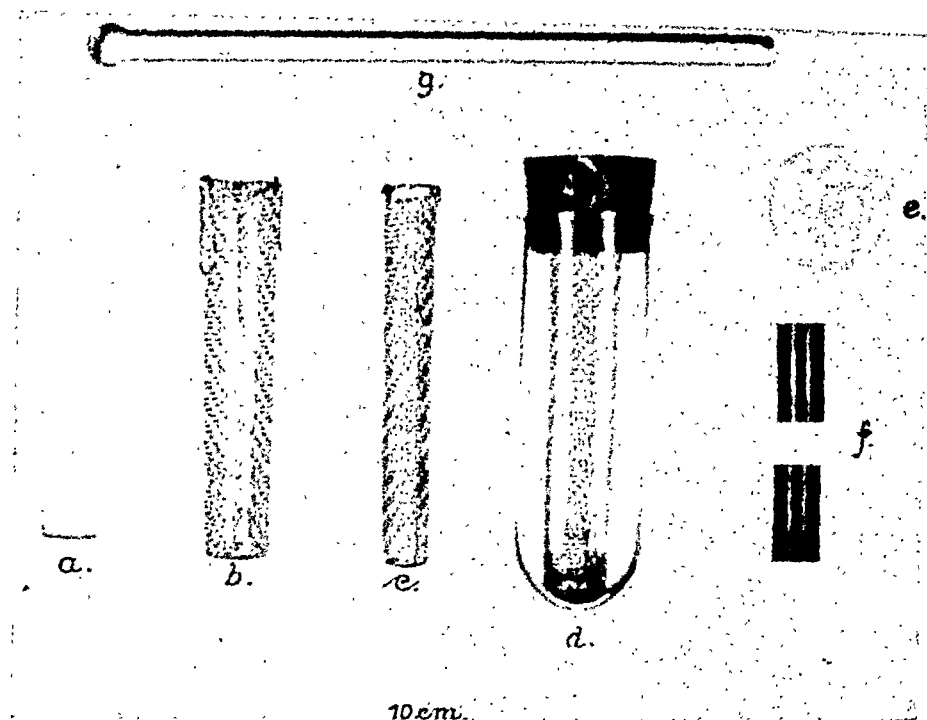


Fig 2.:

a. dry cellophane tubes. b. basket with tube. c. small size basket d. centrifuge tube with basket ready for centrifuging. e. stopper. f. ebonite cylinders. g. glass rod.

sufficient to prevent the tube from collapsing even when it gets soft. It is best, however, to fasten the top of the tube to the basket by means of two paper clips, which are slid over the edges of the tube and the basket (Fig. 2, b). The cellophane tube is now filled with the material to be ultrafiltered and it is placed in an ordinary thickwalled 50 ml centrifuge tube, which is closed with a stopper into which has been cut a circular excavation (Fig. 2, e), fitting tightly over the top of the basket, which in this way is kept centered in the middle of the centrifuge tube (Fig. 2, d). The tubes are centrifuged in an ordinary centrifuge at a speed of 3,000 rev. pr. min. In the centrifuge used in this laboratory the fluid column in the cellophane tube has an average distance from the axis of 10 cm. and the height of the fluid column being about 8 cm. this corresponds to an average filtering pressure of more than 4 atmospheres. The filtering surface of the normal size of tubes is 40—45 sq. cm. and ultrafiltrate is rapidly formed. As the height of the fluid column decreases the filtering pressure is reduced and consequently the filtration rate is diminished.

This may be compensated for by filling in more fluid or if this is not available by filling up part of the cellophane tube by some inert material, whereby the height of the fluid is increased. For this we have used cylinders drilled from ebonite (Fig. 2, f) and having a diameter slightly smaller than the cellophane tubes. If the small size of cellophane tubes is not at hand, these ebonite cylinders may also be used to reduce the capacity of the larger tubes in cases where the volume of fluid available for ultrafiltration is but small. The large size tubes hold about 15 ml., while the smaller hold but 6 ml. Tubes with varying contents of cellulose have been tried, the content being varied by the factory through dilution of the viscose mother solution. Most of the experiments have been carried out with tubes prepared from 8 % solution which is the standard solution of the factory. The tubes prepared with this content are, however, rather impermeable and can only be used in cases where high speed is not essential. The filtering rate of 8 % tubes is from 1 to 4 ml. pr. hour, the variation partly being due to the varying thickness of the tube wall, partly to variations in the drying process. Tubes of 6 % viscose give 2—5 ml. pr. hour, those from 5.5 % 3—6 ml. and those from 5 % 5 to 10 ml. pr. hour.

The tubes made from 8 and 6 % solutions have been absolutely impermeable to protein when tested against egg white and serum, while those from 5.5 % and especially those from 5 % are not absolutely so though they let through only traces of protein. The permeability towards crystalloids has been tested amongst others with glucose and saccharose solutions. The filtrate formed from the 8 % tubes contain 97—99 % of the glucose present in the mother solution and 90—96 % of the saccharose, while the 5 % tubes let through 100 % of the glucose and more than 98 % of the saccharose. This shows that especially the 8 % tubes are so impermeable that regard must be paid to this if the problem studied calls for an exact determination of the filterable part of a substance. If this is the problem it is best solved either by using the most permeable tubes or by a combination of ultrafiltration and dialysis, performed by letting the ultrafiltrate formed during the centrifugation stay in the centrifuge tube in contact with the cellophane tube for some hours, during which time complete equilibration between ultrafiltrate and inner fluid results. If a very high colloid concentration has been obtained in the inner fluid this may, however, under these circumstances

lead to a reabsorption of some of the filtrate. If this is the case the best result is probably obtained by centrifuging at a lower speed, so that equilibration takes place during the centrifugation. When prolonged centrifugation is necessary, it may produce an unwanted heating of the fluid. This is best counteracted by the use of a special centrifuge. A centrifuge, which may be kept at zero degrees, has been in use in this laboratory for some time.

Many problems which do not call for a quantitative separation of the two phases, but where a qualitative separation suffices may be best solved by means of the most impermeable tubes. Problems which usually are solved by means of dialysis may with advantage be tackled by this method. A colloid solution may for instance be freed much more rapidly from a crystalloid component by the use of these tubes than by simple dialysis. In this case the centrifugation is prolonged until but small amounts of the inner fluid are left, when the tube is filled up with some suitable dilution fluid and the centrifugation is renewed; eventually this process is repeated several times. Very high concentrations of the colloid component of a fluid may be reached in the inner fluid, where it is finally found as a concentrated layer on the bottom of the cellophane tube. This like other centrifugation ultrafiltration methods, shows the great advantage over the suction or pressure filtration methods that no clogging of the pores of the membrane occurs, as the concentrated material slides down the walls and collects at the bottom. Various examples of the use of this method, which may be used also with collodion tubes, will be published from this laboratory in the near future.

### Summary.

A centrifugation method for ultrafiltration is described. The essential point of this is the use of factory made cellophane tubes, which when wet become permeable for crystalloids. They are used in conjunction with a basket of metal wire netting. The filtration rate and permeability varies with the cellulose content of the membrane.

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# Sensory Impulses in the Cardiac Depressor Nerves in Experimental Hypertension. A Study of a Problem in Homeostasis.

By

ERNST BÁRÁNY.

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## I. Introduction.

### 1. The Problem.

In his book "The mechanism of nervous action" (1932) E. D. ADRIAN writes:

"We can say if we like that with all sense organs the frequency is determined by the intensity of excitation, though this is merely defining what we mean by intensity of excitation. But the stimulus, the mechanical change inflicted on the sense organ, is *always discounted more or less rapidly by the adaptation which it produces, and the time factor is quite as important as the intensity factor even with the organs which adapt very slowly.*"<sup>1</sup>

This was the starting point of the present investigation, which is an enquiry into the rôle that adaptation might play in reflexory homeostasis of bodily levels.

The multitude of levels maintained with considerable constancy in the higher organisms presumably requires a multitude of automatic regulating arrangements. In fact, as CANNON (1929) points out, it is reasonable to look for a special regulator as soon as a level seems to be rather constant. These regulators presumably could act according to various principles, utilizing overflow mechanisms, chemical equilibria etc. There are good reasons

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<sup>1</sup> Italics by present author.

to suppose, however, that some of them are arranged as reflex mechanisms beginning with a sense organ directly or indirectly stimulated by the level to be controlled. The question now is what would happen if such a sensory ending showed adaptation to its stimulus.

Reflectory regulation must always function according to one fundamental principle, *viz.* that decrease of receptor stimulation changes the level in the more stimulating direction. Thus, e. g. adaptation of the thermoreceptors presumably controlling our body temperature would lead to slowly rising temperature if they were heat receptors and slowly decreasing temperature if they were cold receptors. (If both kinds of receptors were engaged, the direction of drift would be determined by their relative adaptation rates.) If blood pressure were controlled by baroreceptors in the carotid sinus and elsewhere, adaptation of these would cause a drift of blood pressure towards higher levels, etc., etc.

Now, nothing is known of a temperature drift with age. There is, however, an increase of blood pressure with age even if it is an open question if this is a really physiological phenomenon. Anyhow, constancy of these levels is incompatible with adaptation in the controlling receptors. Either, the receptors do adapt but are not really determining the regulated level; (they might act only as shock absorbers, trying to readjust the level to whichever value it has possessed a long enough time — a possibility suggested for the carotid sinus by GAMMON 1936); or, they do adapt very slowly and this is one of the causes of the blood pressure rise with age; or, the receptors do not adapt indefinitely but keep up a discharge proportionately to the controlled level for an unlimited time. The present work, which was started in 1938, is an attempt to study these possibilities for the special case of blood pressure. Ideas very similar to those expressed above have been put forward by THAUER (1939) in a review on the thermoregulation of the body, but no decision is arrived at or even attempted.

## 2. Previous Work on the Carotid Sinus Baroreceptors Bearing on Our Problem.

The case of blood pressure is a special one insofar as the stimulus is pulsatile. Thus, even if the end organs did show complete adaptation to the mean blood pressure, the pulse synchronous

flow of depressor impulses from the receptors might go on. Thus, we cannot deduce the non-existence of adaptation from experiments (e. g. HEYMANS and BOUCKAERT 1939) showing permanent rise of blood pressure after denervation of the carotid sinuses and aortic region nor draw the opposite conclusion from experiments with opposite results (e. g. GOLDBLATT, KAHN, BAYLESS and SIMON 1940 and many others).

Nor is it possible to draw any definite conclusions from the fact that compression of the carotids in dogs with renal hypertension sometimes gives much the same blood pressure response as in normal cases (VERNEY and VOGT 1938) or an augmented response (BOUCKAERT, ELAUT and HEYMANS 1937). The same is true of similar experiments on man (MANDELSTAMM and LIFSCHITZ 1932, GAMMON 1936 and many others). Obviously, the blood pressure response to carotid compression may depend upon many other factors than the actual flow of impulses from the sinus and these factors certainly might be different in normals and hypertensives.

Some information could be obtained from experiments with isolated carotid sinus preparations. If adaptation were relatively rapid, the blood pressure response to a steady pressure in the isolated sinus would decrease with time. In fact, this has been noted and ascribed to either adaptation or deterioration by KOCH (1931). There remains, however, the possibility that this escape of the blood pressure from depression is an escape of the sympathetic centers from inhibition (BRONK 1933—34). STRAUSS (1940) reports that it is less if pulsatile instead of continuous pressure is used, a finding which he ascribed to slower deterioration with pulsatile pressure. In unpublished experiments the author (1938) sometimes observed no signs of adaptation to steady pressure in the isolated carotid sinus during as long as 45 minutes. In these experiments not the blood pressure level as such was used as index for the impulse flow from the sinus, but the rise of blood pressure on sudden removal of the intrasinusoidal pressure. This has the advantage that changes in the general circulation of the animal, for instance a progressive rise or drop of blood pressure are not mistaken for changes in the impulse discharge from the preparation. The blood pressure response to sudden pressure drop in the isolated sinus is fully developed in less than 1 minute, during which short interval there is little chance for spontaneous blood pressure changes to occur.



The experiments showed that the blood pressure response may be the same on removal of a steady pressure after 45 seconds and after up to 45 minutes. This, of course, is true only in that minority of cases where the preparation is undamaged after so long a time. In fact, the viability of the preparation sets so narrow limits to the period of observation that only relatively rapid adaptation (from our point of view) would be possible to detect in such experiments. The same limitation must to a still higher degree hamper attempts to solve the problem by observation of the impulse response from single isolated end organs (BRONK and STELLA 1934—35). In the summary these authors write: "The discharge continues indefinitely at a frequency but slightly less than the initial maximum value. Thus these receptors are of a type that adapts but little."

In another paper BRONK (1934) writes: "It is interesting to observe — and important for the purposes of our discussion — that there are marked differences in the rate and in the degree of adaptation of different types of sense organs. Thus muscle spindles — — — tension receptors in the root of the lungs — — — pressure receptors in the arteries — — — and pain receptors in the cornea — — — adapt relatively little and do that slowly — — — a rapidly adapting pressure receptor in the wall of the carotid sinus would not faithfully signal the level of blood pressure. A slowly adapting sense organ is needed for following the variations in intensity of a long-lasting stimulus."

It is not always clear, however, for how long the authors have followed the discharge. The longest observation period mentioned or illustrated by BRONK and STELLA (1934—35) is only 8 seconds. According to a personal communication quoted by GAMMON (1936) BRONK has seen a maintained discharge for periods of several minutes. It is obvious, however, that with this technique observation periods of more than at most a few hours would be impossible.

Summarizing the existing evidence: it is shown by BRONK and STELLA (1934—35) that the baroreceptors show some initial adaptation. Other experiments have demonstrated that the latter course of adaptation, if existent, is so slow that it cannot be detected in experiments covering only minutes or hours. In the experiments described below an attempt has therefore been made to give adaptation more time to develop, in fact, many hundreds to thousands of hours.

## II. Methods.

### 1. Main Principles.

The main principle of the experiment consists in a comparison of the impulse discharge from the baroreceptor endings in normal and chronically hypertensive animals *at the same blood pressure*. Comparison at the same blood pressure is essential for the following reason: Assume for an instant that some adaptation, however incomplete, takes place in the hypertensive animals. At their raised blood pressure they will then still have more impulses in the depressor nerves than normal animals at their normal pressure. As we do not know the law according to which the impulse number depends on the mean pressure we cannot tell if that number is raised proportionately or not in the hypertensive cases. If, on the other hand, all blood pressures are reduced to a common standard level at the moment of comparison, any adaptation in the hypertensive cases will reveal itself as too low an impulse frequency.

The intensity of discharge of the baroreceptor endings might conceivably be judged either by the impulse frequency in isolated single fibers or by the total impulse discharge in a group of fibers. Obviously, in the case of single fibers, random sampling effects concerning threshold, viability etc. would play a major rôle. Therefore, it was decided to try to evaluate the whole nerve discharge. This would seem to be rational also from other points of view. According to BISHOP, HEINBECKER and O'LEARY (1934) "in responses to stimulations of the depressor nerve of the rabbit in preparations uncomplicated by the presence of pressor effects, the curves of action potential area against stimulus strength and of per cent depression of blood pressure against stimulus strength, recorded coincidentally, have a similar form". Thus, there would seem to exist at least a rough parallel between the total discharge of impulses and the vasomotor effect, and as we wish to study the impulses as possible causes of vasomotor effect it seems rational to use the action potential of the whole nerve as an index of activity.

### 2. Choice of Experimental Animal.

Clearly, very great technical difficulties would meet an attempt to use the carotid sinus baroreceptors in the present study, once it has

been decided to use the action potential of the whole nerve. As using a large series of dogs would have been out of the question for economic reasons, small laboratory animals had to be used. Here, the difficulties in preparing the sinus nerve in such a manner as to be sure not to have damaged it, even slightly, seemed prohibitive. Fortunately, the rabbit cardio-aortic depressor nerve lends itself ideally to our purpose. As it runs isolated from the vagus it is relatively easily dissected for a long enough stretch.

### 3. Production and Control of Hypertension.

Systolic blood pressure was measured in the central artery of the ear by a modification of the method of MOERS and SCHLIENZ (1939). By using a transparent plastic for the apparatus its construction can be much simplified.

As is well known, measurements in this artery are very dependent upon the temperature of the animal, only animals kept well warm having dilated ear arteries. As waiting for spontaneous dilatation in some 25—50 animals can take much time and denervation of the ear has no lasting effect the ears to be used were vigorously rubbed with xylol half a minute before the measurements. This treatment effects a maximal dilatation of the artery and very considerably reduces the time and labour necessary for controlling the blood pressure. When frequent blood pressure estimates are made, most of the rabbits eventually become so used to the procedure that no resting period is necessary before the measurement.

Hypertension was produced by a combination of PAGE's cellophane perinephritis (1939) and contralateral nephrectomy. Rabbits of a highly inbred strain and of approximately the same age were used. At a weight of about 1,200 gm. the rabbits were operated on for the first time and a cellophane capsule applied around the left kidney. Autoptic controls showed that cellophane loosely wrapped around the kidney as recommended by PAGE (1939) did not regularly produce tightfitting scar tissue capsules, but was often broken up, leaving most of the kidney free. Therefore, small cellophane pouches were tied around the kidney, care being taken not to compress the hilus. Stay sutures were not usually made and no harm was seen to result from this omission.

After about three weeks a very moderate blood pressure rise occurred, which showed, however, a tendency to disappear. Therefore about one month after the first operation the right kidney was removed. Immediately after this the blood pressure used to increase some 30 mm. and to remain at this level. Fig. 1 shows a typical example of the systolic blood pressure response to the operations. In this case, which died from an intercurrent intestinal infection, an unusually large number of blood pressure determinations were made in order to bring out clearly the steps in the blood pressure curve.

Probably the mechanism behind the rapid blood pressure rise after the second operation is the same as in the experiments of GREEN-

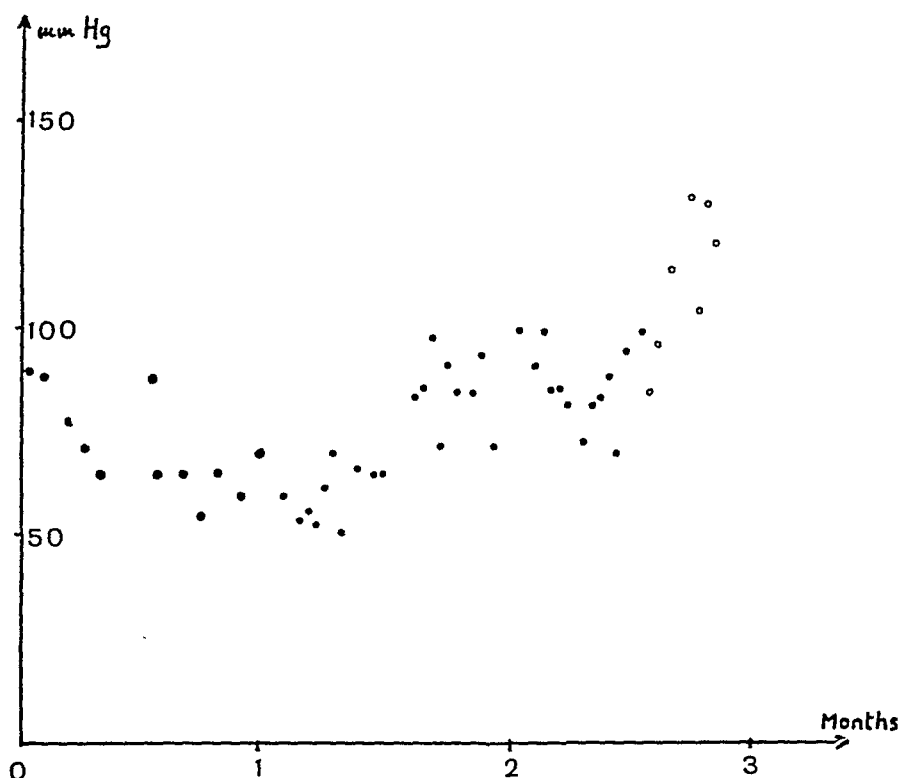


Fig. 1.

Development of hypertension.

Ordinate: Systolic blood pressure in central ear artery. Large dots: before operation. Small dots: after encapsulation of left kidney. Circles: after removal of right kidney. All blood pressure values are the mean of at least three determinations in immediate succession.

WOOD, NASSET and TAYLOR (1939) using a collodion cast around one kidney and extirpating the other. The authors believe it to be prevention of hypertrophy. It would seem more probable, however, in view of the rapidity of the blood pressure rise, that the encapsulated kidney is compressed because it must accommodate more blood when doing the work of two.

#### 4. Recording of Action Potentials.

Action currents in the cardio-aortic nerves were recorded diphasically with silver — silverchloride electrodes 6 mm. apart leading to a Toennies differential amplifier, followed by two straight resistance — capacity coupled stages and a cathode ray oscillograph. The recording camera paper speed was about 50 cm./sec. It was frequently controlled by introduction of 50 c. p. s. voltage in the circuit and did not vary appreciably. A power amplifier with loudspeaker branched off before the cathode ray driving stage.

### 5. Stabilization of Blood Pressure.

The arrangement for stabilization of blood pressure is illustrated in fig. 2.

Oxygen from a bomb passes along the overflow arrangement *O* to the wide-area reservoir *R* filled with 0.002 % heparin in RINGERS

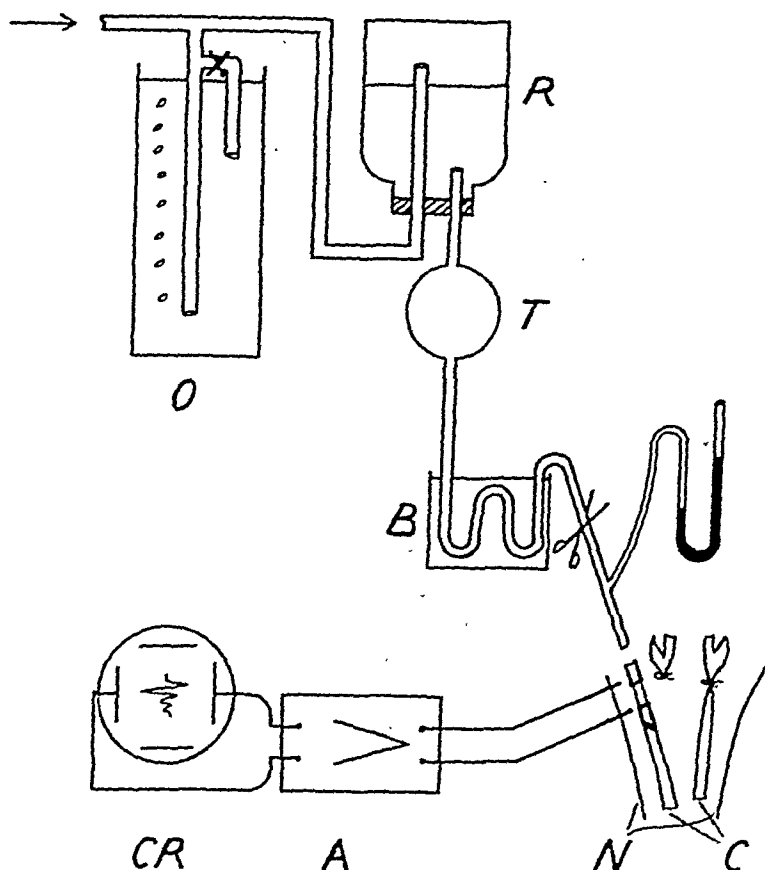


Fig. 2.

Arrangement for main experiment.

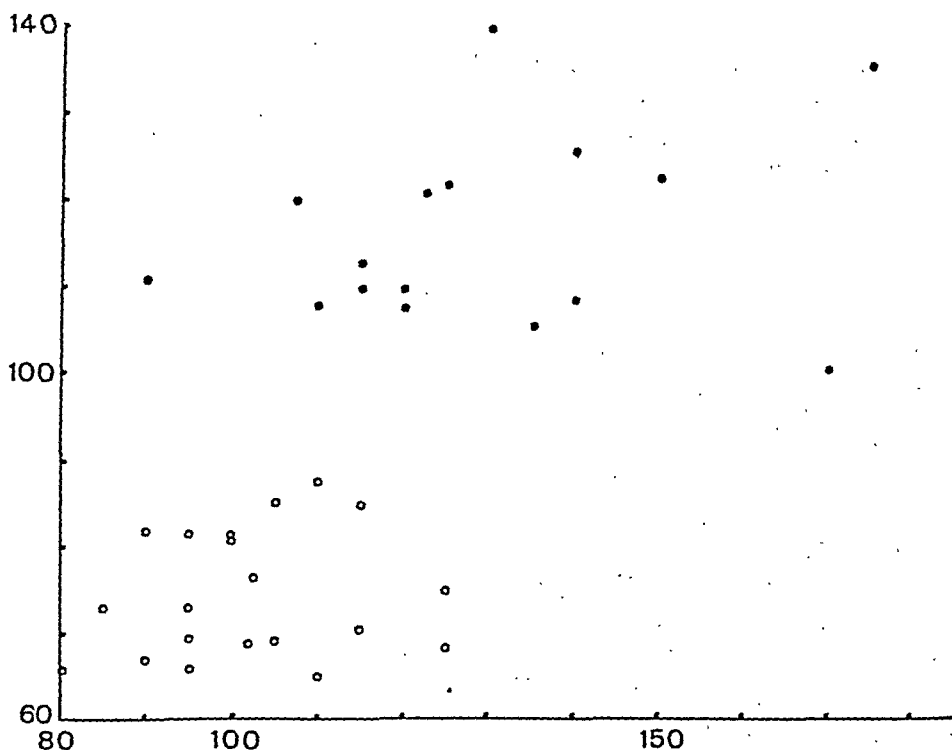
A amplifier, B 37° C water bath, C common carotid arteries, CR cathode ray oscillograph, N cardio-aortic nerves, O overflow for gas pressure stabilization, R reservoir, T blood trap.

solution. The reservoir is connected with a blood trap *T* leading to a hot-water bath *B*. From *B* a rubber tubing goes to a mercury manometer and the carotid cannula. This rubber tubing may be compressed near the manometer branch so as to reduce the arrangement to a conventional blood pressure set up. The overflow arrangement can be set to two predetermined pressure levels in the artery cannula, in our experiments 58 and 98 mm. Hg. All glass surfaces which come into contact with blood are covered with a film of ferric stearate.

### 6. Course of Complete Experiment.

The course of a complete experiment was as follows. The rabbit — normal or hypertensive — had blood pressure readings taken the last 5 days. It was narcotized with 1.5 g/kg urethane intravenously. The preparation was made with the utmost care and always performed in the same manner: after median incision through the praelaryngeal muscles and tracheotomy, a silk is tied around the superior laryngeal nerve and by slight traction with the silk the angle between the superior laryngeal nerve and the vagus is exposed. Here the cardio-aortic nerve originates, usually with one root from each of the angle forming nerves but sometimes from only one of them. In a few cases the nerve left the vagus some 5 mm. below the angle. Sometimes a free cardio-aortic nerve was lacking altogether on one side. A fine silk strand is tied as a handle around the suspected nerve and this is cut above the knot. Then, to prevent accidental haemorrhage, the large thyroid artery is tied and divided, care being taken not to tie it too close to the carotid but preferably some 3 mm. distally. After this is done, the whole operating field is filled with plenty of lukewarm Ringer and half a minute let pass. The connective tissue swells enormously in the Ringer, in fact it is almost solved. Even rather tough fascia becomes a jelly with the consistence of warm butter and it is easy to free the fine nerve from most of the overlying fascia without any violence. A somewhat dangerous point is where the nerve passes below the thyroid artery. In the angle between the carotid and that branch there is usually some fat and the connective tissue around it seems not to be reduced to jelly as easily as elsewhere. If the ligature around the thyroid is made too near the carotid, the nerve may be drawn into the knot or at least maltreated by traction in adjacent connective tissue. Here and elsewhere the nerve is finally cut free for some 30 mm. with fine scissors and placed curled up in a safe, moist and warm corner of the operating field. It is wise to place the nerve on one of the electrodes as soon as some 5 mm. are dissected free in order to control by the loudspeaker that the correct nerve is under work.

When both nerves are dissected free the blood pressure cannula is introduced in the right carotid after heparinization with 5 mg. heparin/kg. Care is taken not to exert any traction on the distal or central part of the vessel. The stabilizing arrangement remains excluded and a reading of bloody blood pressure is taken. This will be higher than in the intact animal because of exclusion of the cardio-aortics and one carotid sinus, but it still bears a relation to the unbloody mean pressure determined before the experiment. (Fig. 3.) After the reading is taken, the left carotid is ligated leaving the animal almost without moderator reflexes. (A slight reflux via the vertebral arteries may reach the carotid sinuses but is without practical importance as are the reflexes from baroreceptors in other regions.) Then the tubing to the blood pressure stabilizer, set at 98 mm. Hg, is released. Usually some blood leaves the circulation and within a few minutes the mean blood pressure has settled around 98 mm. This is controlled by com-



### 7. Evaluation of Records.

The cardio-aortic nerve in the rabbit seems to convey baroreceptor impulses in two main groups of fibers giving large and small pulse-synchronous spikes respectively. In this it resembles the sinus nerve of the cat (EULER, LILJESTRAND and ZOTTERMAN 1941). The chemoceptor impulses from the aortic body obviously travel in a third group of very fine caliber, the spikes of which are not visible in records from the whole nerve. This was checked partly by attempts to discover small diastolic spikes at low blood pressures, which was always futile, partly by comparing records at constant blood pressure with and without hyperventilation with oxygen. There was never any differ-

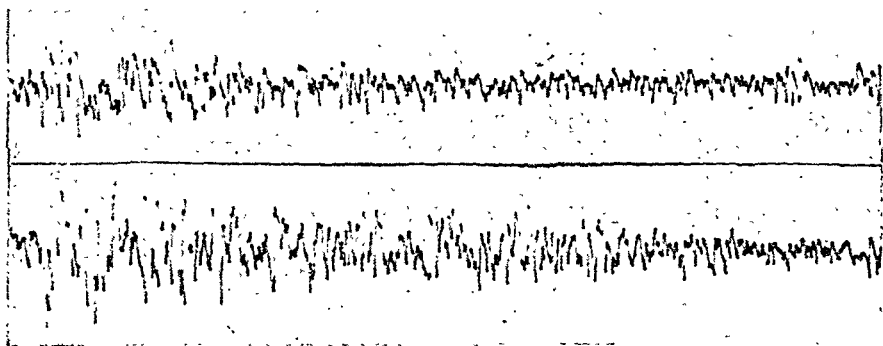


Fig. 4.

Records from the same depressor nerve at 58 (above) and 98 mm. Hg mean blood pressure. To be read from left to right. Scale 1 : 1. Paper speed 50 cm/sec.

ence between the records. Thus, it is not necessary to attempt a separation between chemoceptor and baroreceptor impulses, the main visible discharge being baroreceptor. For reference to pressor fibres in the depressor nerves (O'LEARY, HEINBECKER and BISHOP 1934) see below.

In a first attempt at evaluation, two observers independently counted everything in the records that resembled a spike. The part of a record counted always began with the start of the first complete systole and ended with the end of the last complete diastole. The results of the two counters occasionally differed a good deal but in the mean proved nearly identical and surprising: The impulse frequencies were the same at blood pressures of 58 and 98 mm. The reason for this is clear from a look at fig. 4, showing a record at 58 and one at 98 mm. blood pressure from the same nerve. With rising pressure, there appears a rising number of large spikes which mask the small spikes, making them impossible to discern and count. This masking action is so disturbing that we reluctantly had to give up the idea of counting all spikes and had to confine the counting to the large impulses. It is true that, as EULER, LILJESTRAND and ZOTTERMAN (1941) point out, the small spikes may be as important from the reflex point of



view as the large ones. There is, however some justification for our procedure too. The paper by BISHOP, HEINBECKER and O'LEARY (1934) already quoted shows that the total action current area, which, to judge from our records, usually would be a function mainly of the frequency of large spikes, goes parallel with the vasomotor effect. The authors write: "— — the effect per impulse varied as the action potential area per stimulus for fibers of different size. If area of potential varies as cross-sectional area of fibers, then the central effect per fiber varies as fiber mass."

After we had decided only to count the large spikes, a new difficulty arose, namely: which spikes were to be counted as large? The amplitude of a spike is a function of the fiber diameter and the diameter of the nerve with sheath and fluid layer. This latter diameter is different in different nerves. Thus, even with constant amplification, it would be impossible to delimit the large spikes only from their absolute magnitude. It is always necessary to define them by comparison with small spikes. Fortunately, the groups differ so much that it is usually easy to distinguish between members if the record is not too dense. At the end of systole, this usually is the case and the separation always started here. Not all records, however, did show marked diastolic frequency minima. Feeling that this might depend on spontaneous activity from injured fibers, we discarded all records from a nerve with one such record. This also should eliminate nerves with many pressor fibers (O'LEARY, HEINBECKER and BISHOP 1934) as there is no reason to believe that these fibers are activated only during systole.

Some of the spikes counted as "large" might be synchronized "small" ones. As this, however, depends upon chance, it will happen with equal frequency in both groups of animals if the spike densities are equal and thus it does not matter from our point of view.

For the final treatment, records of all kinds were mixed and the counter instructed to look upon each record as an isolated problem, determining the limit for large impulse magnitude for each, without reference to the other records from the same nerve. Of course, the records were not marked in such a way as to show which kind they belonged to. In the large pool of records there were two from each nerve obtained at 98 mm. Hg, one of them from the beginning and one from the end of the experiment. Thus, placing of the nerve on the electrodes and equilibration of the blood pressure were independently remade for the second of them. As the records were counted quite independently, their differences could be used for a calculation of the random error of the whole method. Analogously, by counting a series of records twice, the random error of the counting process was determined (cf. below, p. 17). The main systematic error is probably caused by the mutual masking of densely grouped spikes. Thus, certainly, all values are too low and this the more the higher they are. There is no reason to believe, however, that this error affects normal and hypertensive animals differently and thus it is of small importance for the present investigation.

### III. Material.

Allowing for mortality and subsequent losses from various reasons, only some 30 % of the about 100 animals present from the start could be used. The number of normal controls was 16 with 31 usable depressor nerves, the number of hypertensives was 19 with 34 usable nerves. The mean weight of the normal group was 2.26 kg., that of the hypertensive group 2.22 kg. The mean systolic blood pressure during the whole control period was 78 mm. Hg for the normals, while mean systolic blood pressure during the last week before the main experiment was 118 for the hypertensives. The distribution of the blood pressures can be seen in fig. 5. The duration of hypertension calculated from the date of the second operation was 57—93 days, with the median at 78 and the mean at 77. In addition, two animals with hypertension lasting 253 and 335 days were used. They were survivors from a large series which perished from intestinal infection a short time after the second operation. The effect of the hypertension may be judged from the resulting hypertrophy of the hearts. The heart weights in grams per kg. body were 2.2 in the normals and 2.6 in the hypertensives, the corresponding mean absolute heart weights 5.0 and 5.8 grams. (Unfortunately about 50 % of the hearts were not weighed.)

### IV. Results.

#### 1. The Main Result.

After these lengthy preliminaries, the main result can be concisely expressed: No longtime adaptation was discernible. Table I and fig. 5 *a* and *b* bring this out clearly. They show the relation between previous mean systolic blood pressure and impulse frequency (large spikes/millisecond) at constant mean blood pressure. Circles refer to nerves from normal animals, dots to hypertensives. Numerically, the results are summarized in table I. The figures show that previous long time blood pressure level has no detectable influence on the impulse frequency.

It is extremely improbable that the result can be explained by other assumptions, which will, however, now be discussed.

The hypertensive animals lost more blood to the stabilizer than did the controls. As they could hardly have a higher blood

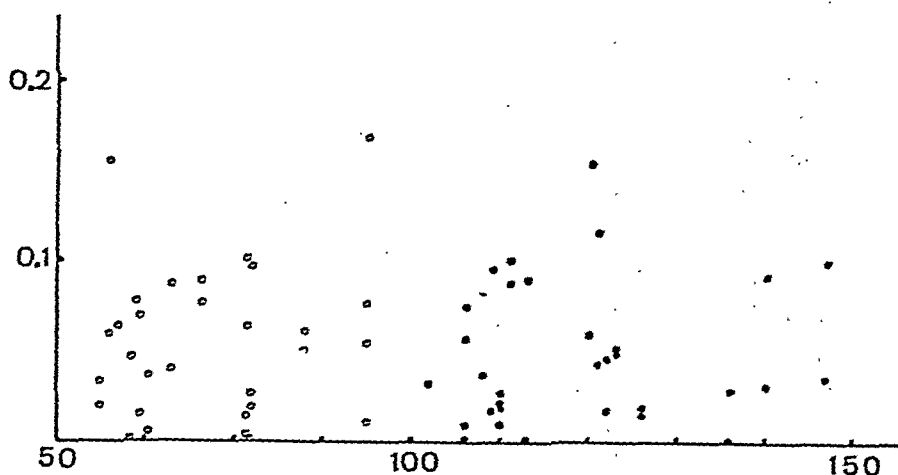


Fig. 5 a.

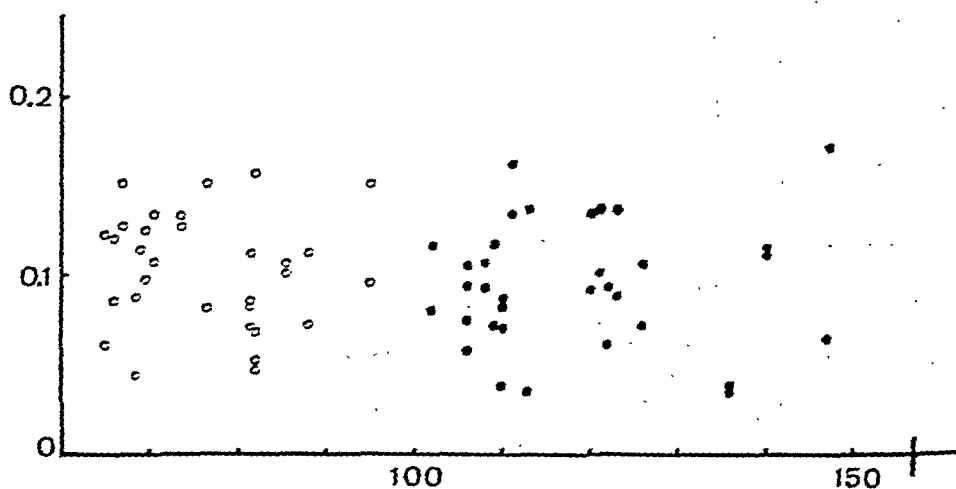


Fig. 5 b.

Fig. 5.

Relation between impulse frequency and previous average systolic blood pressure. Ordinate: impulse frequency in large spikes/millisecond. Abscissae: previous average systolic blood pressure. Dots: hypertensives. Circles: normals.

5 a: Blood pressure stabilized at 58 mm. Hg, each point one determination.

5 b: Blood pressure stabilized at 93 mm. Hg, each point the mean of two independent determinations.

volume to start with their vessels must have been more contracted during the experiment than those of the controls. Thus, differences in the form of the central pulse responsible for the discharge from the cardio-aortic baroreceptors cannot be excluded. On the other hand, the connection with the manometer is equal in normal and hypertensive cases and should tend to equal-

lize conditions. Moreover, the pulse form has no or at least no great influence on the blood pressure reflex and therefore presumably not on the mean impulse frequency. As BÁRÁNY (1942) has shown, superposition of even rather violent sinusoidal pulsations upon a steady mean pressure in the isolated carotid sinus has no influence on the blood pressure. The experiments of STRAUSS (1940) show a slight influence of nonsinusoidal pulses, but his experimental conditions are open to criticism (BÁRÁNY, loc. cit.). Anyhow, changes in the pulse form would *mainly* change the distribution of the impulses during the heart cycle and, obviously, such redistribution has no important influence, the centers exerting a kind of fly-wheel action. STRAUSS did not find any influence of pulse frequency on the blood pressure reflex. Thus, the very slight and statistically insignificant differences in rapidity of heart beat between normal and hypertensive animals (Table 1) can be of no importance.

Table I.

	Normals (1)	Hypertensives (2)	Difference (1)–(2)
Large spikes/millisecond.			
at 58 mm Hg . . . . .	$0.055 \pm 0.007$	$0.051 \pm 0.007$	$0.004 \pm 0.010$
at 98 mm Hg . . . . .	$0.103 \pm 0.006$	$0.097 \pm 0.006$	$0.006 \pm 0.009$
Duration of cardiac cycle in millisec.			
at 58 mm Hg . . . . .	$253 \pm 5$	$263 \pm 5$	$-10 \pm 7$
at 98 mm Hg . . . . .	$250 \pm 5$	$262 \pm 5$	$-12 \pm 7$

Having excluded the possibility of physical differences between normals and hypertensives being of importance, we have to consider possible chemical differences. One obvious chemical difference is the occurrence in one group of the renal products responsible for the hypertension. It is hard to believe, however, that these substances could act so as to mask an adaptation. They would then have to increase the impulse discharge from the baroreceptors, thereby depressing the blood pressure. Moreover, the baroreceptors seem to be very insensitive to changes in their chemical environment. This was shown for  $O_2$  and  $CO_2$  by BRONK and STELLA (1934–35) and for synaptotropic substances by EULER, LILJESTRAND and ZOTTERMAN (1941). In unpublished experiments, the author could detect no difference in the blood pressure reflex from the isolated carotid sinus when the calcium

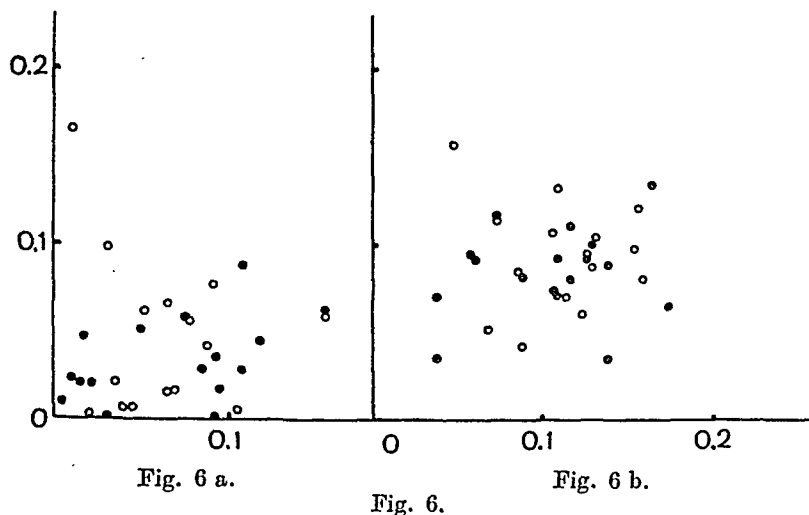
content of the perfusion fluid was halved or doubled. Thus, differences in the chemical milieu can hardly be believed to mask adaptation.

There further remains the remote possibility that the recent excitation history of the baroreceptors could blot out the effects of previous stimulation so as to equalize the discharge from end organs with normal and hypertensive history. Adaptation is a change of threshold. Equality in response to the same stimulus despite differences in previous stimulation could be an indication either of extremely slow or lacking adaptation as we have previously assumed. But it might also be caused by an adaptation proceeding so rapidly that the threshold of the receptors returns to normal in the preparatory stage of blood pressure equalization. Now, all previous experience quoted above indicates that adaptation of the depressor baroreceptors is slow. An independent proof is given by the fact that the impulse frequency at 98 mm. is approximately the same in the records taken before blood pressure reduction to 58 mm. and immediately after cessation of that reduction. In fact, the impulse frequencies before and after that reduction were for the normals  $0.099 \pm 0.007$  and  $0.107 \pm 0.007$  large spikes/millisecond and for the hypertensives  $0.105 \pm 0.009$  and  $0.089 \pm 0.007$ . The differences are obviously insignificant and moreover of different sign. Thus, it can be accepted as certain that the immediately previous history of stimulation has no influence on the impulse frequency and that the experiments prove that even very long stimulation has no such influence, that adaptation is absent or extremely slow.

## 2. The Correlation between Right and Left Nerves and the Question of the Statistical Unit.

As most of the animals participated with 2 nerves in the material, the question arose if animals or nerves were to be counted as statistical units. The answer depends upon the degree of correlation between impulse frequency in the right and left nerve.

Fig. 6 *a* and *b* shows the correlation between impulse frequencies in right and left nerves *at the same blood pressure for all animals*, which eliminates an important factor tending to give positive correlation, *viz.* the identical blood pressure for both nerves. The diagram shows that there can be no question of a strong correlation and in fact the BRAVAIS-PEARSON coefficient turns out to be less than 0.1. Thus, it is permissible to treat the single



Relation between impulse frequencies in right and left nerve.  
 Ordinate: impulse frequency in large spikes/millisecond in left nerve.  
 Abscissæ: same in right nerve. Dot: hypertensives. Circles: normals.

6 a: Blood pressure stabilized at 58 mm. Hg, each point one determination

6 b: Blood pressure stabilized at 98 mm. Hg each point the mean of two independent determinations.

nerve as the statistical unit, as has been done in the preceding section.

### 3. The Importance of Random Errors versus Interindividual Variability.

In order to be able to judge the results, it is necessary to have an idea of the causes of the large scatter characterizing the material. This scatter will be caused partly by the random errors of the recording and counting methods and partly by real interindividual variability. Both may be expressed in the forms of standard errors. The standard error of the method is obtained from the above mentioned series of pairs of records at 98 mm. Hg, recorded and counted independently (p. 12). There are 64 such pairs and when treated according to DAHLBERG's method (1940) they give a value for the standard error of the whole method for one single counted record of  $\sigma_M = 35\%$ . Analogously, the error of the counting method as calculated from the series counted twice becomes  $13\%$ . The interindividual variability  $\sigma_I$  is obtained from the methodical variability  $\sigma_M$  and the total variability  $\sigma_T$  according to the formula

$$\sigma_I = \sqrt{\sigma_T^2 - \sigma_M^2}.$$

$\sigma_T$  is  $43\%$  as obtained from all normal and hypertensive records at 98 mm., the calculation being performed for each group separately and the weighted mean of the results taken.

This makes the interindividual variability  $\sigma_I = 25\%$ .

Obviously, the error of the method for a single determination is rather large, being larger than the interindividual variability. Therefore, when possible, the mean was taken between the two counted records from the same nerve at the same pressure. This reduced the error of method to the same magnitude as the interindividual variability, namely to 25 %. Thus in fig. 5 *b*, the scatter depends equally on methodical imperfections and real interindividual variability and even with an ideal method the width of the band would be more than 70 % of that obtained now. There would still be no significant difference between normals and hypertensives.

## V. Discussion.

### 1. Significance of a Negative Result.

It is a deplorable fact that the non-existence of an effect cannot be rigidly proved even by statistical methods. Thus, the present investigation only shows that the decrease of impulse frequency at a normal blood pressure caused by adaptation to a blood pressure some 50 % above normal, if really existent, is small in comparison with the interindividual variability, being  $6 \% \pm 9 \%$ . The following discussion is based on the very probable assumption that this difference is caused merely by chance and that, consequently, no long time adaptation is present. It is however possible that a very slow adaptation takes place. This might be the cause of the c:a 2 mm. Hg per annum rise in blood pressure observed in man.

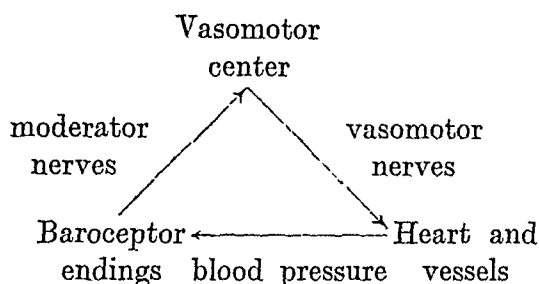
### 2. Significance of the Small Spikes in the Depressor Discharge.

With the reservation made above, our results may be said to show that the large cardio-aortic fibers carry afferent impulses giving a faithful record of the mean blood pressure. We have no means to decide if the same is true of the small fibers. If their endings behave in the same manner as those of the large fibers, their importance might equal or exceed that of the latter, as proposed by EULER, LILJESTRAND and ZOTTERMAN (1941). If, on the other hand, their endings behave differently, not giving a true record of the blood pressure, the centers will have to discriminate against them in order to avoid confusion. They then might still play the main part in a shock-absorbing mechanism, a possibility which nevertheless seems rather remote.

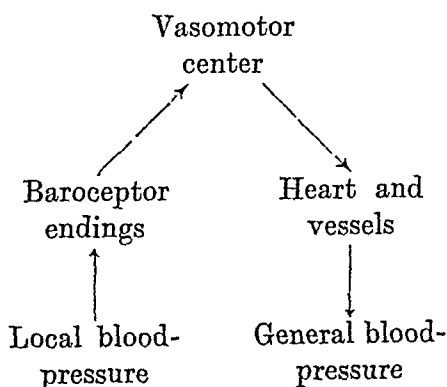
### 3. Significance of Our Results for the Understanding of Renal Hypertension.

We begin with a short consideration of the behaviour of a selfcontrolled system such as the blood-pressure regulation.

Schematically this can be represented by the well-known triangle



Thus, we have three interdependent organs connected by links of various nature: two nerve paths and the blood stream. Now, the question is: how and at which level does stabilization occur? In order to analyze the action of the system one has to cut one of the links, opening the circle of interaction. This can be done anywhere, for instance in the blood stream link. This implies isolating the baroreceptive endings from the general circulation, a procedure which is not completely possible experimentally but feasible for purposes of argument. We then have a *local* blood pressure acting on the baroreceptors and a *general* blood pressure controlled by the state of these baroreceptors. The diagram becomes



By varying the local pressure we can change the general pressure. The functional relationship may be represented by curve



A in fig. 7. Now, at which blood pressure does the system become stable when connected up in the original state? Obviously at the point where local blood pressure and general blood pressure are equal, that is at the intersection between curve A and the  $45^\circ$  line through the origin. This is true also if any permanent changes have taken place in the system. Such changes, e. g. in the viscosity of the blood, sensitivity of the baroreceptors, of the vasomotor centers etc., will take the form of changed functional relationship between local and general blood pressure as shown

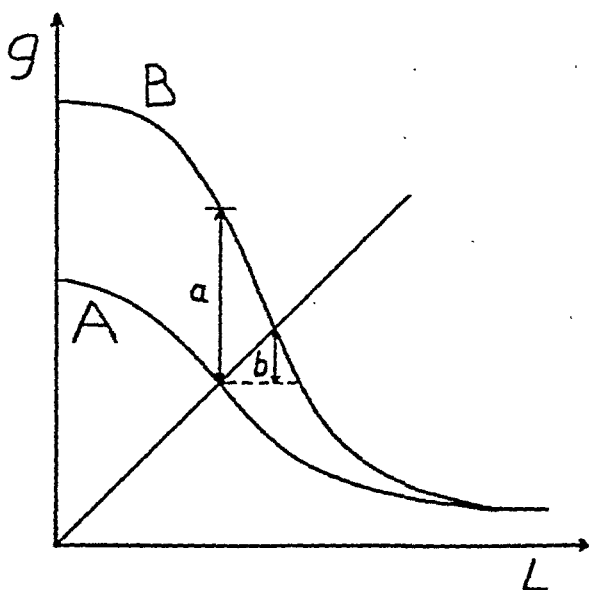


Fig. 7.

G = general blood pressure, L = local blood pressure. Further explanation see text.

for instance by curve B in fig. 7. It is seen that the blood pressure rise caused by the supposed change without concomitant change of local blood pressure, that is with the moderator nerves out of action, would equal the distance *a* while the introduction of the moderators reduces the change to only *b*. A change always remains, however, even with the moderator apparatus intact — a fact which is not always realised.

Thus Dock (1940) has shown in a valuable contribution

1) that the peripheral vascular tonus in GOLDBLATT hypertension is dependent upon concomitant central vasomotor tonus and is completely removable by pithing.

2) that in these animals the vessels are sensitized to epinephrine and presumably to adrenergic nervous impulses.

Because of the existence of the moderator apparatus he believes that the sensitization of the vessels to adrenergic impulses is not alone able to produce hypertension. He therefore concludes that a changed "set" of the centers is necessary. But, as has been demonstrated above, sensitization alone would necessarily change the equilibrium level of blood pressure, as indeed would any change in the system that could permanently change the functional relation between local blood pressure in the baroreceptive regions and general blood pressure. *Not the vasomotor center but the whole system as such determines the equilibrium level*, every link in the chain is equally important. Therefore, DOCK has not shown that the centers are in any way altered in GOLDBLATT hypertension — which does not exclude the possibility that they might be so.

The construction shown in fig. 7 may be used for estimating how large an increase in equilibrium level might be caused by the  $6\% \pm 9\%$  decrease of impulse frequency, at about normal blood pressure, found in our hypertensives. Assuming a rough proportionality between impulse frequency and local blood pressure (as borne out by table I), the change (if real) of the endings means a  $6\% \pm 9\%$  shift of the curve towards the right. The point of intersection will then be displaced *less than*  $6\% \pm 9\%$  upwards along the ordinate axis, because of the slope of the curves. Thus, the actual hypertension, some 50 %, cannot be explained by a change in the endings.

### Significance of Our Results from the Point of View of End Organ Physiology.

The present study reveals for the first time that there are end organs which are capable for hundreds or even thousands of hours of faithfully signalling the stimulus level. What this means for the theory of homeostasis is evident from the introductory part of this paper. In order to judge the general significance of our finding for the physiology of end organs, it is necessary to consider in how far the pulsatile nature of the stimulus could be the cause of the sustained discharge from the endings. Is it possible that only the opportunity for recovery offered in each diastole makes it possible for the ending to keep up the discharge? In order to answer this question with certainty one would want quantitative facts about the behaviour of slowly adapting single end organs under pulsatile stimuli. No such facts are available however. The interesting studies of CATTELL and HOAGLAND (1931) on intermittent stimulation of rapidly adapt-

ing skin receptors have no bearing on our problem. What we need are studies where mean strength of stimulus and amplitude of pulsation are controlled as separate parameters. Without such information, any conclusion on the above points can only be tentative. The following argument is offered in this spirit.

In his fundamental paper, MATTHEWS (1931) has shown that the adaptation after rapid reloading of a partially adapted muscle spindle proceeds in two steps, one rapid and one slow. (Fig. 18, MATTHEWS 1931). MATTHEWS explains this by the assumption that two processes in the ending antagonize adaptation, a slow *a*-process of large impulse capacity and a rapid but rapidly exhausted *b*-process. During a short recovery pause only the *b*-process recovers appreciably and the heightened response after a short recovery is therefore of short duration, the response drops very soon almost to the level obtained before the recovery pause, which is mainly determined by the *a*-process. Thus, it is perhaps conceivable that, from an adapted end organ, a pulsatile stimulus would at each pulse only release a number of impulses, belonging mainly to the rapid *b*-process, adaptation (or exhaustion) of the *a*-process being complete or almost complete. The discharge would then be governed by the amplitude of pulsation rather than by the mean stimulus level. Now, the present findings do not point in this direction and thus could perhaps be expressed by stating that the slow, *a*-controlled phase of adaptation is lacking in at least some aortic baroreceptors, that their *a*-process is inexhaustible. That a rapid *b*-phase does exist in kindred receptors is shown by the initial adaptation demonstrated in the carotid sinus by BRONK and STELLA (1935). The impulses released by the pulse beat and belonging to the *b*-process cannot, however, be very important from the depressor point of view, as shown in the studies of the influence of pulsations on the carotid sinus reflex by STRAUSS (1940) and BÁRÁNY (1942). Thus, these studies give indirect evidence of the lack of *a*-adaptation.

### Summary.

1. Reflectory homeostasis can only be effected by means of nonadapting sensory endings reporting the controlled level to the centers.

2. At the same blood pressure the large fibers of the cardio-aortic nerves of rabbits with experimental renal hypertension

of a few months standing carry a number of impulses that is not significantly less ( $6\% \pm 9\%$ ) than in normal controls. Thus, the baroreceptor endings in the aorta connected with the large depressor fibers either lack progressive adaptation or adapt at an extremely slow rate.

3. The aortic baroreceptors are not involved in the production of renal hypertension of the GOLDBLATT type.

4. There is no correlation between the impulse frequencies in the two cardio-aortic nerves, besides that caused by common blood pressure.

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## The Nature of the Amino Nitrogen of Red Corpuscles.

By

HANS H. USSING.

(Received 4 February 1943.)

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### Introduction.

It has been known for a long time (CONSTANTINO 1913) that amino acids or substances reacting like amino acids are present in a higher concentration in the red corpuscles of mammals than in the surrounding plasma. Similar conditions are found in the case of several other substances, as for example magnesium, phosphoric acid<sup>1</sup> and creatine. Regarding the amino acids there is, however, the complication that the amount of amino acid in the corpuscles is dependent on the concentration in the plasma. It is clear therefore that it is not sufficient to assume that the corpuscles are impermeable to amino acid.

Several attempts have been made to elucidate this phenomenon, but none of the hypotheses has as yet proved satisfactory.

ABDERHALDEN and KÜRTE (1921) were of the opinion that the corpuscles adsorb amino acids on their surface, and that this adsorption follows FREUNDLICH'S adsorption equation. Several authors have denied that the uptake of amino acids by red corpuscles is in accordance with this equation (SBARSKY 1923). HÄUSLER (1926) was of the opinion that the adsorption after some time is transformed into an equal partition. SBARSKY (1941)

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<sup>1</sup> Using radioactive phosphate HEVESY et al. (vid. HAHN and HEVESY 1942) have shown that phosphate is diffusing at a reasonable rate through the corpuscle membrane. The apparent surplus of phosphate in the corpuscles originates from unstable phosphate esters, for instance creatine phosphate, which are constantly being formed and broken down.

points out that the equilibrium is reached rather slowly, which speaks against an adsorption to the corpuscle surface. He speaks therefore of a "sorption", a combination with colloids in the interior of the corpuscles. FOLIN and SVEDBERG (1930) regarded the surplus of amino acid found in the corpuscles as an artefact produced by some amino acid-like substances split off during haemolysis. DANIELSSON (1933) found that the corpuscles were freely permeable to amino acids. According to him the excess was not amino acids, but substances reacting in a similar way. Without giving any proof of his assumption he supposed that half the surplus was in reality glutathione.

A possibility, which has not been mentioned in the literature, but which cannot be ruled out a priori, is that the amino acids are taken up by the red corpuscles by some active cell-function. It is known (DANOVSKY 1941) that human corpuscles loose  $K^+$  in the cold and take up it again at 20—40°C.

As will be seen from this brief review, a reinvestigation of the whole problem seems justified. The present paper represents an attempt at such reinvestigation.

My thanks are due to Professor KROGH for his constant interest in my work. I also wish to express my thanks to Mrs. KAREN SCHAUFUSS, who has assisted me with the analyses.

### Experimental.

For the experiments fresh ox-blood with the addition of 1 g sodium oxalate per liter was used. The separation of the red corpuscles and the plasma was performed by centrifuging for half an hour at 3500 rev./min. This separation is not complete, the corpuscle fraction containing about 10 % plasma. The error introduced in this way is in most cases insignificant for the interpretation of the results. In one single series of experiments where this source of error is of importance a suitable correction will be used.

The determination of the amino-N content was performed by the method of VAN SLYKE (1929) on protein-free filtrates from corpuscles and plasma.

Two different procedures for the preparation of these filtrates were used. One sort of filtrates were ultra-filtrates, prepared according to the method of REHBERG (1943). It is possible to use this method even on undiluted corpuscles if they have been previously haemolysed by repeated freezing. The ultra-filtration was performed in a cooled centrifuge, the temperature being kept all the time below 6°C. The ultra-filtrate thus prepared contains mainly those constituents of the corpuscles or the plasma the molecular-weight of which are below 1000.

The other type of filtrates were prepared after a modification of the method of LUCK (1928) for the determination of amino acids in tissues. One ml plasma or one ml corpuscles diluted in the proportion  $\frac{1}{2}$  by weight was measured into a graduated test-tube containing 5 ml water, faintly acidified with acetic acid and previously heated to 100°C on a steambath. The test-tube remained in the steambath for seven minutes and was shaken at intervals. After cooling one ml. 20 % trichloroacetic acid was added after which the tube was filled up to the 10 ml mark and well shaken. After standing for half an hour one g kieselgur was added and after shaking the fluid was filtered through a dry filter into a cylinder divided to 0.1 ml. The filtrate was evaporated in a porcelain dish until only 3 ml remained to remove most of the trichloroacetic acid. Then the fluid was made alkaline with NaOH and boiled for one minute to remove  $\text{NH}_3$ . Upon this the fluid was neutralized with glacial acetic acid. The resulting fluid (1—2 ml) was transferred into the van Slyke apparatus so that the analysis + wash-water measured 5 ml. Then the analysis was performed as usual.

The treatment with kieselgur according to LUCK (l. c) removes some peptides, which are not precipitated by trichloroacetic acid, so that all substances left in the filtrate which react with  $\text{HNO}_2$  are true amino acids. As will be shown later this assumption is not correct.

#### a) The Normal Content of Amino-N in Corpuscles and Plasma.

Table I shows the amino-N content of ultra-filtrates from corpuscles and plasma. For filtrates prepared according to LUCK the results are calculated under the assumption that the amino acids are dissolved in the water-phase only, which for the corpuscles may be estimated to 60 % of the corpuscle weight. For the plasma the waterphase is taken to be 92 %. Calculated on this basis the determinations on the two different sorts of filtrates may be compared. It is seen that the agreement is fairly good, not only as regards the plasma but also as regards the corpuscles.

Table I.

	Ultra filtrate mg % amino-N	"Luck" filtrate mg % amino-N
Corpuscles . . . . .	19.3 19.7	19.4 19.4
Plasma . . . . .	4.6 5.3	4.5 5.8



As the filtrates prepared according to LUCK certainly contain all amino acids present in the corpuscles and since the ultra-filtrates are showing just as high a content of amino-N, we can conclude that *corpuscles which are haemolysed by freezing do not adsorb amino acids*.

#### b) Influence of Temperature on the Uptake of Amino Acid.

37 mg leucine was added to 3 blood-samples, of 50 ml each, after the blood-samples through a short centrifuging had been separated into corpuscles and plasma. In this way the leucine could be dissolved in the plasma without producing haemolysis. Upon this the samples were mixed well and placed at 0°, 20° and 37° respectively. Before the centrifuging all the blood-samples had been brought to the temperature at which they were later incubated.

At suitable intervals samples were drawn, centrifuged and the amino-N content of the corpuscles determined. Table II shows the result. As will be seen the uptake of leucine increases with temperature and with time.

Table II.

	mg % amino-N in corpuscle ultra-filtrate after	
	5 hours	22 hours
0° . . . . .	—	24.4
20° . . . . .	21.4	26.5
37° . . . . .	25.7	32.0

This result speaks against the assumption of an adsorption of amino acids by the corpuscles, as a positive adsorption will always decrease with temperature.

#### c) The Uptake of Different Types of Amino Acids.

The amount of leucine used in the experiments just mentioned should give an increase of about 10—12 mg% amino-N under the assumption that whole blood has a dry substance content of 18—21 % and that the added amino acid is distributed evenly in the water-phase. It is therefore interesting that the concentration

of amino-N in the water-phase of the corpuscles approaches 32 mg%, which is an increase of just about 12 mg%. The uptake of amino acid by the corpuscles corresponds to a diffusion to an equilibrium. In order to investigate if all amino acids behave in the same manner similar experiments were performed with tyrosine, alanine, phenylalanine, glutamic acid and aspartic acid. For the four last mentioned amino acids the same method as for leucine was used. The amounts of amino acid added to the blood-samples were in each case equivalent with 37 mg leucine. Tyrosine was determined as such on ultra-filtrates from corpuscles and plasma with a quantitative modification of the Millon reaction (ARNOW, 1937). Table III shows the result. After 24 hours at 37° added

Table III.

*15 mg tyrosine was added to 50 ml blood. The blood was placed at 37° for 24 hours.*

	Colour value, arbitrar units			increase in mg % tyrosine
	— tyrosine	+ tyrosine	increase	
Corpuscle ultra-filtrate . .	50	276	226	24.2
Plasma ultra-filtrate . . .	30	290	260	27.8

tyrosine is evenly distributed between corpuscles and plasma ultra-filtrate. The results for the other amino acids is seen from table IV. It will be seen that alanine and phenylalanine behave like tyrosine and leucine, whereas but little increase is seen in the case of glutamic acid and aspartic acid. If this increase is cor-

Table IV.

	mg % amino-N in corpuscle ultra-filtrate after 24 hours at 37°	
	without added amino acid	with added amino acid
Alanine . . . . .	19.8	29.0
Alanine . . . . .	19.0	30.3
Phenyl alanine . . . . .	19.4	30.2
Glutamic acid . . . . .	19.4	22.3
Aspartic acid . . . . .	19.2	21.4

rected for the increase in amino-N of the plasma left between the corpuscles it is doubtful whether the two acid amino acids are taken up at all. As previously mentioned about 10 % plasma is left between the corpuscles. This plasma is not removed by centrifuging at the rate used (3500 revolutions/min.). Even when 6000 revolutions/min. is used a residue of 4 % plasma is left between the corpuscles. The plasma contains about 92 % water whereas the corpuscles contain 60 % water. The water-phase from the corpuscles contains accordingly 14 % water originating from the plasma. If the plasma concentration of amino-N in the experiments with glutamic acid and aspartic acid is increased about 14 mg% amino-N, the amino-N content of the corpuscles will be  $14/100 \times 14/100 = 2$  mg% too high.

It looks as if the corpuscles are practically impermeable to dicarbonic amino acids. The hexon-bases have not been studied as to their diffusion into the corpuscles, but in view of the low permeability of corpuscles towards cations it is probable that they will diffuse slowly if at all. These considerations lead to the assumption that the substance or the substances responsible for the high amino-N concentration of the corpuscles is to be sought amongst charged, acidic or basic, amino acids or amino acid-like substances.

#### d) The Effect of Acid Hydrolysis on Corpuscle Filtrates.

Although the trichloroacetic acid filtrates were treated with kieselgur which according to LUCK (l. c.) removes polypeptides and the ultra-filtrates were not likely to contain higher peptides on account of the relatively small pores of the cellulose-membrane used, it seemed advisable to perform a hydrolysis of the filtrates to ascertain whether any peptide substance should be present. For the hydrolysis hydrochloric acid was used. Equal parts of the filtrates in question and concentrated HCl were mixed in test tubes which were afterwards sealed and heated for 18 hours at 110° in an electric oven.

After cooling the total content of each tube was transferred to a porcelain dish and evaporated on a steam bath to a small volume. It was then made distinctly alkaline and boiled for 2 minutes to remove ammonia. After neutralisation an  $\text{NH}_4$ -determination was made either on the total sample or on an aliquot part. The results of such experiments are shown in table V. It is seen that the





To the alkaline solution 100 ml conc. hydrochloric acid was added and the mixture was heated on a steam bath for 2 hours to hydrolyse the pyrrolidone carbonic acid. At all stages of the procedure aliquot parts were taken out for amino-N determination. See table VII.

Table VII.

	mg N	mg % N in water phase
Filtrate before heating . . . . .	15.2	19.2
Filtrate after heating . . . . .	5.4	6.9
Alkaline extract before hydrolysis . . .	0.7	0.9
Alkaline extract after hydrolysis . . . .	4.2	5.5
"Glutamic acid" N . . . . .	3.5	4.6

Other experiments gave similar results. In one case the glutamic acid chlorhydrate was prepared. Though the yield was poor the crystals could be identified by their shape and the parallel extinction in polarized light.

It was, however, obvious that glutamic acid could be responsible for at most a third of the excess amino-N of the corpuscles. At least one more substance, the amino-N of which disappeared on heating near the neutral point must be present in the corpuscle filtrates.

#### f) The Amount of Monamino Monocarbonic Acids in Corpuscle Filtrate.

In a previous section of this paper it was shown that added monamino monocarbonic acids diffused into the corpuscles until the increase in amino-N was equal on both sides of the corpuscle membrane.

Even when no amino acids are added the sum of monamino monocarbonic acids on either side of the membrane would probably be equal. The exact amount of these amino acids in the plasma is not known; probably the corresponding amino-N content amounts to about 2—3 mg%. An estimate of these amino acids in the corpuscles may be obtained by removing the basic amino acids (+ cystine and certain peptides) with phosphotungstic acid and the dicarbonic amino acids (+ tyrosin) by saturation with Ba(OH)<sub>2</sub> and precipitation with alcohol in excess.

Example: A filtrate from 200 ml corpuscles and 1,800 g water was prepared in the same way as described in the preceding section with the following alterations viz. the corpuscles were washed with 0.9 % NaCl before precipitation and 100 g kaolin was used instead of 50 g kieselgur to remove impurities. The filtrate (1,220 ml) was concentrated in vacuo, and the concentrated solution (50 ml) was freed from trichloroacetic acid by ether extraction and again concentrated to 10 ml. It was then washed into a centrifuge tube with 2 ml water and 1 ml conc. HCl. The tube was heated in a water bath to 90° and 3.5 g phosphotungstic acid dissolved in 2 ml hot water was added under stirring. After standing for 48 hours in the cold the precipitate was centrifuged and washed with  $2 \times 2$  ml icecooled 2 % phosphotungstic acid in 4 % HCl.

The combined mother liquor and washings were freed from phosphotungstic acid by extraction with amylalcohol-ether (compare BLOCK 1934) and the solution was concentrated to 10 ml and stirred with pulverized baryum hydroxide until saturation was reached. A precipitate which contained the remaining trace of phosphotungstic acid was centrifuged, washed with 5 ml saturated  $\text{Ba}(\text{OH})_2$  and discarded. The solution + wash water was poured under stirring into a centrifuge tube with 75 ml 96 % alcohol.

After standing in the cold for 48 hours the precipitate was centrifuged and washed with 70 % alcohol and 98 % alcohol.

The original amino acid mixture was thus divided into three fractions: 1) The phosphotungstic acid precipitate. 2) The baryum salts of the dicarbonic acids. 3) The alcoholic solution containing mainly the monamino monocarbonic acids. The phosphotungstic precipitate was dissolved in dilute NaOH which was afterwards neutralized with acetic acid.

The baryum salts of the dicarbonic acids were dissolved in water and the last trace of alcohol was removed by concentration in vacuo. The alcoholic solution of the monamino acids was diluted with water and concentrated in vacuo until all alcohol was removed.

All fractions were filled up to the mark in measuring flasks and the amino-N content was determined on aliquot parts. The results of such experiments are shown in table VIII. It is seen that the monamino monocarbonic acids are present in the concentration which would be expected if these amino acids are diffu-

Table VIII.

	First experiment mg %	Second experi- ment mg %
Total amino-N . . . . .	15.0	14.6
Amino-N in phospho-tungstic precipitate	9.0	9.3
Amino-N in Ba-precipitate . . . . .	2.6	2.2
Amino-N of monamino acids . . . . .	2.1	1.4

sing freely through the corpuscle membrane. Of course the figures should not be taken too literally. The fraction supposed to consist of monamino acids may contain traces of the other sorts of amino acids which may have escaped precipitation and on the other hand a certain amount of monamino acids may have been lost. Nevertheless it is obvious that no amino acid of this group plays any part in producing the excess amino-N of the corpuscles.

The amount of amino-N which is precipitated by phosphotungstic acid is astonishingly high; but perhaps the most interesting feature is that the dicarbonic acid fraction is so low that glutamic acid cannot possibly be of any importance in producing the excess amino-N. The interest was now focussed on the substance or the substances which were precipitated with phosphotungstic acid. The substances precipitated might be hexon-bases, cystein and certain peptides especially glutathione.

#### g) The Amount of Glutathione in Corpuscle Filtrates.

As mentioned above DANIELSSON (l. c-) suggested that about half the indiffusible amino-N of the corpuscles came from glutathione.

It was therefore necessary to determine the amount of this tripeptide in the corpuscle filtrates.

The determinations were carried out according to the method of FUJITA and NUMATA (1938 a). This method involves the reduction of oxidized glutathione with  $H_2S$ , removing of  $H_2S$  by evacuation, and titration in an ice cooled vessel at  $p_H$  2 of the reduced glutathione with iodate after addition of iodide in excess. In the original method ascorbic acid is oxydized by ascorbic oxydase; but according to FUJITA and NUMATA (1938 b) the amount of ascorbic acid in blood is so small that practically all the reducing



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power towards iodine in acid solution is due to glutathione. The treatment with oxydase was therefore omitted in the present investigation. For the determinations ultra-filtrates and metaphosphoric acid filtrates (1 part corpuscles + 9 parts 4 % metaphosphoric acid) were used. Trichloroacetic acid filtrates according to FUJITA and NUMATA (1938 b) contain  $\text{Fe}^{+++}$  which interferes with the titration. The results are shown in table IX.

Table IX.

	Glutathione mg %	Calculated amino- N from glutathione, mg %	Total amino-N mg %
Corpuscles, metaphosphoric acid filtrate . . . . .	124	8.9	23.8
Corpuscles, ultra-filtrate . . .	120	8.6	24.3
Corpuscles suspended in Ringer for 24 hours, metaphosphoric acid filtrate . . . . .	124	8.9	22.4
	92	6.6	19.8
	85	6.1	16.4

### Conclusion and Discussion.

The increase in amino-N during acid hydrolysis (part d) of corpuscle filtrates seemed to indicate that a tripeptide, for instance glutathione, could be present in an amount corresponding to at most 3—4 mg% amino-N. Yet the direct determination of glutathione shows an amount corresponding to 6—9 mg% N in free amino groups. The explanation of this discrepancy is found in the paper of HOPKINS (1929) in which the right formula for glutathione is set forth. Glutathione gives namely much more than one molecule N for each molecule glutathione with nitrous acid in the van Slyke apparatus. Indeed this peculiarity, according to HOPKINS, was one of the main reasons why glutathione was first regarded as a dipeptide.

HOPKINS found that glutathione yielded somewhat more than half its total N in the van Slyke apparatus. A sample of glutathione prepared in this laboratory from red corpuscles according to the directions of HOPKINS (l. c.) gave 1.9 moles N for each mole glutathione. This factor seems to fit well with the above statement of HOPKINS.

This means that glutathione accounts for a still greater part of the amino-N found in corpuscle filtrate than the amount calculated from the free amino groups of the tripeptide. Further it is clear that when glutathione gives nearly two moles N determined directly the increase in amino-N on hydrolysis will only be one mole N. A closer calculation leads indeed to the result that if the amino-N determined for glutathione is subtracted from the total amino-N, the amount left is of much the same magnitude as the amino-N of the plasma.

Example: A sample of metaphosphoric acid filtrate from corpuscles gave 124 mg% glutathione in the corpuscle water phase. The corresponding amount of amino-N in free amino groups is 8.9 mg%. Each mole of glutathione giving 1.9 moles N, the amount of N originating from glutathione must be  $8.9 \times 1.9 = 17$  mg%. The total amino-N of the sample was 22.4 mg%, difference 5.4 mg%. In the corresponding plasma the amino-N content was 5.5 mg%.

The same result may be gained in a partly independent way. Under the assumption that every mole of glutathione gives three moles amino acid on acid hydrolysis, which according to HOPKINS (l. c.) runs smoothly, the difference between the total amino-N after hydrolysis and the calculated amount of amino-N derived from the glutamic acid, cystein and glycine formed during hydrolysis should be equal to the amino-N of the plasma.

Example: A sample of corpuscle ultra-filtrate gave on hydrolysis 24 mg% amino-N the glutathione content was 92 mg% corresponding to  $3 \times 6.6$  mg% = 19.8 mg% amino-N after hydrolysis. Difference 4.2 mg%, which fits well with the plasma amino-N, 5.1 mg% or corrected for urea destroyed during hydrolysis 4.1 mg%.

When using the last mentioned method it should be considered that cystine gives somewhat too high results in the van Slyke apparatus. On the other hand some cystine is destroyed during hydrolysis and the same holds true for certain other amino acids. The sources of error are thus likely to balance each other.

In section *e* it was shown that the greater part of the amino-N of corpuscle filtrates disappeared on prolonged heating near the neutral point. According to HOPKINS (l. c.) it is peculiar to glutathione that it is totally destroyed under such circumstances, and the main split products: pyrrolidone carbonic acid and the diketopiperazine of glycine and cysteine give no  $N_2$  in the van Slyke apparatus. The fact that only 5—7 mg% amino-N is left after

heating corpuscle filtrates for 48 hours at neutral reaction strongly supports the view that the corpuscles are containing real amino acids in the same concentration as in the plasma and that all the excess amino-N originates from glutathione. Most of the glutamic acid found in the corpuscle filtrates (section *c*) originated undoubtedly from glutathione.

In section *f* it was found that a large proportion of the amino-N of the corpuscle filtrate is precipitated by phosphotungstic acid. This is in accordance with the fact that glutathione is precipitated by phosphotungstic acid. It is, however, somewhat soluble in excess of the precipitant (HOPKINS 1921) and it was therefore to be expected that some glutathione escaped precipitation. As glutathione is strongly adsorbed by baryum phosphotungstate the glutathione still in solution would be removed before the precipitation of the dicarbonic acids.

The phosphotungstic acid fraction consisted accordingly of most of the glutathione + the hexon-bases which are probably not present in higher concentration in the corpuscles than in the plasma.

It will be seen that there is strong evidence for the view that practically all excess amino-N in the corpuscles originates from glutathione.<sup>1</sup>

Of course the methods used are not able to detect very small amounts of some other substances simulating amino acid; but it is improbable that such substances should be responsible for more than 1 mg% amino-N.

Further it must be remembered that although the sum of true amino acids in the corpuscle filtrates and in the plasma are alike this does not necessarily mean that the same is the case for each separate amino acid. In view of the relative ease with which monamino monocarbonic acids pass through the corpuscle membrane (section *b* and *c*) no difference can be maintained in the case of these amino acids. The dicarbonic amino acids owing to their slow permeation through the corpuscle membrane might well be present in different concentrations on either side of the membrane perhaps for several days.

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<sup>1</sup> As mentioned above DANIELSSON (l. c.) supposed that about half the indiffusible amino-N originated from glutathione. He gives no experimental proof of his view. Probably his estimate of the amount of glutathione is too low because most earlier methods determined only part of the glutathione. FUJITA and NUMATA (1938a) have shown that the reduction with Zn which has been in common use destroys a considerable part of the glutathione.

The hexon-bases have not been examined as to their diffusion rate into the corpuscles, but in view of the general low permeability towards cations the rate is probably low.

As to the permeability to amino acids the corpuscles of different animals show considerable variation (compare DANIELSSON (l. e.)).

It still remains to be explained how the high concentration of glutathione is maintained in the corpuscles. The most reasonable explanation apparently is that the corpuscles are so impermeable to glutathione that the glutathione present in the newly formed corpuscles is more or less completely kept back during the whole lifetime of the corpuscles. In this connection it is of interest that glutathione is a dicarbonic acid of about the same strength as glutamic acid (PIRIE and PINKEY, 1929) which was shown above to diffuse very slowly through the corpuscle membrane. Glutathione may thus be looked upon as a molecule too large to pass through the pores in the membrane and on account of its dicarbonic acid nature it is insoluble in the lipoid phase of the membrane.

There is of course the possibility that glutathione is continuously synthesized in the corpuscles. If this were the case it would be very interesting because most syntheses seem to be dependent on a cell nucleus. WAELSCH and RITTENBERG (1941) have proved by the aid of amino acids labelled with heavy N that glutathione is synthesized at a very high rate in most living cells but unfortunately the red corpuscles were not examined so that it still remains a question if the corpuscles of the circulating blood are able to synthesize glutathione.

The third possibility that the corpuscles might take up glutathione from the plasma can be ruled out with some certainty, because the plasma does not contain measurable amounts of glutathione. Indeed according to OBERST (1935) the plasma contains some substance which destroys glutathione.

The fact that glutathione is responsible for practically all the indiffusible amino-N of the corpuscles implicates that nearly all amino acid determinations hitherto made on whole blood are erroneous. FOLINS method for amino-N determinations on un-laked blood (1930) gives probably the best approximation to the right value, but as the different true amino acids in the corpuscles are diffusing out more or less slowly and some of them extremely slowly the results are almost certainly too low. Apparently no

method is as yet known which removes glutathione quantitatively without removing one or more amino acids. It seems therefore preferable to make amino-N determination on the plasma which contains no glutathione.

Most tissues contain glutathione in still higher concentrations than does the corpuscles and the amount of amino acid found in the tissues by different investigators (compare for example LUCK (l. c.)) is undoubtedly too high. Experiments now in progress in this laboratory indicate that although glutathione is responsible for a considerable proportion of the amino-N of the tissues, true amino acids are still present in higher concentration in the tissues than in the blood plasma.

### Summary.

The permeability of red corpuscles to amino acids is examined. It is shown that monamino monocarbonic acids diffuse until an even partition is reached in the two water phases. This process takes less than 24 hours at 37°. The dicarbonic acids: glutamic acid and aspartic acid are diffusing extremely slowly through the corpuscle membrane. It is demonstrated that monamino monocarbonic acids are present in about the same concentration in the corpuscles and the plasma.

The origin of the high amino-N content of the corpuscles compared with the plasma is investigated and it is found that nearly all the excess amino-N came from glutathione so that the concentration of true amino acids is equally high in the water phases of corpuscles and plasma.

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## Über die Oestronwirkung auf den Sauerstoffverbrauch normaler, hypophysenloser und hypophysenstiieldurchtrennter Kaninchen.<sup>1</sup>

Von

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(Eingereicht am 12. Februar 1943.)

Der Einfluss des Oestrone auf den Grundumsatz normaler Tiere ist mehrfach untersucht worden, aber mit wechselndem Resultat. So fanden KUNDE et al. (1930) bei Hunden keine Änderung des Grundumsatzes, ABBA und MERLINI (1939) bei Kaninchen keine sichere Veränderung, bei erwachsenen Meerschweinchen jedoch eine Tendenz zu einer Steigerung. DANFORTH, GREENE und IVY (1937) sahen bei Ratten ebenfalls keinen sicheren Effekt, während GESSLER (1936) bei Meerschweinchen, GRUMBRECHT und LOESER (1938) bei Ratten und SHERWOOD (1940) bei Ratten und Kaninchen nach der Oestronzufuhr eine Senkung des Grundumsatzes feststellten.

Bei hypophysenlosen Ratten und Meerschweinchen wurde der Grundumsatz nach Oestronzufuhr von GESSLER (1937) unverändert gefunden.

Der Sauerstoffverbrauch stiieldurchtrennter Tiere ist bisher nicht untersucht worden. Die histologischen Befunde von BROOKS (1938), WESTMAN und JACOBSON (1938, 1940), UOTILA (1939, 1940) und WESTMAN, JACOBSON und OKKELS (1941) an den Hypophysen und Schilddrüsen stiieldurchtrennter Ratten und Kaninchen deuten jedoch auf eine Störung der Produktion thyreotroper Hormone und lassen eine Änderung des Grundumsatzes nach der Stiieldurchtrennung erwarten.

<sup>1</sup> Diese Untersuchung wurde mit Unterstützung der Stiftung »Therese und Johan Anderssons minne» Karolinska Institutet, Stockholm, ausgeführt.

Da man, wie WESTMAN und JACOBSON (1942) zeigen konnten, die nach der Stieldurchtrennung in der Hypophyse auftretende Zellatrophie durch langdauernde Oestronzufuhr rückgängig machen kann, erschien es ferner von Interesse, den Sauerstoffverbrauch mit Oestron behandelten, stieldurchtrennter Tiere zu untersuchen, um festzustellen, ob es vielleicht gelingt, auf diese Weise eine Funktionsänderung des vom Stiel getrennten, nach der Oestronzufuhr normal aussehenden Hypophysenvorderlappens nachzuweisen.

Um ein Vergleichsmaterial zu erhalten und im Hinblick auf die erwähnten widersprechenden Resultate früherer Untersucher wird in der vorliegenden Arbeit der Sauerstoffverbrauch sowohl normaler, wie hypophysenloser und hypophysenstieldurchtrennter Kaninchen vor und nach Zufuhr von Oestron untersucht.

Zunächst werden die Resultate der unbehandelten stieldurchtrennten Kaninchen mit denen der unbehandelten normalen und hypophysenlosen verglichen. Danach wird geprüft, ob sich der Sauerstoffverbrauch der normalen, hypophysenlosen oder stieldurchtrennten Tiere durch die Oestronbehandlung geändert hat.

### Methodisches.

Für die Untersuchung wurden fast ausschliesslich Tiere benutzt, die mehrere Monate unter denselben Ernährungs- und Umweltbedingungen gestanden hatten. Die Bestimmung des  $O_2$ -Verbrauches geschah in Morphin-Urethannarkose (5 mg Morphin hydrochl. + 0.5 g Urethan pro kg K. G. i. m.) unter sorgfältiger Konstanthaltung der Körpertemperatur. Die Messung wurde mit der von KAHLSON und PEIL (1938) angegebenen Apparatur am spontan atmenden Kaninchen vorgenommen. Hierbei wird das Tier durch eine Trachealkanüle mit einem geschlossenen System verbunden, das darin vorhandene Luftgemisch durch ein Gebläse in Strömung gehalten und die vom Tier ausgeatmeten Gase durch einen Natronkalkturm geleitet. Die Messvorrichtung besteht aus einem geeichten Kroghschen Spirometer, dessen Ausschläge auf einer berussten Trommel registriert werden.

Die meisten Tiere hatten sich nach  $1\frac{1}{2}$  Stunde soweit an die Apparatur gewöhnt, dass mit der Messung begonnen werden konnte. Die Atmung war dann regelmässig, und die Spirometerkurve fiel annähernd geradlinig ab. Ein vollkommen gleichmässiger Kurvenfall war bei den dann ca. 1—2 Stunden andauernden Messungen meist nicht zu erreichen. Für die Berechnung wurden deshalb nur die geraden Kurvenstücke benutzt. Die in den Tabellen angegebenen Zahlen sind die Mittelwerte mehrerer Perioden von ca. 10 Minuten.



Für die Oestronbehandlung benutzten wir das Präparat Ovex, Leo. In den meisten Fällen wurden 0.1 mg in 0.1 ccm der Öllösung des Oestradiolmonobenzoat täglich subcutan injiziert.

### Material.

Ausgeführt wurden im ganzen 46 Messungen, von denen 10 unberücksichtigt geblieben sind, entweder weil die Tiere in Pernoctonnarkose oder die Kurven nicht befriedigend waren. Das bearbeitete Material besteht also aus 36 Messungen, die an 21 Tieren vorgenommen wurden. Es wäre natürlich am besten, dasselbe Tier für möglichst viele Messungen vor und nach der Behandlung zu benutzen. Dieses konnte jedoch nicht konsequent durchgeführt werden, da einige Tiere nach dem Versuch Tracheitiden oder Pneumonien bekamen und für weitere Versuche untauglich wurden.

In der Tabelle I bis IV sind die Untersuchungen gruppenweise zusammengestellt. Alle Tabellen sind in der gleichen Weise aufgestellt. Der  $O_2$ -Verbrauch in ccm/10 Min./kg ist vor und zu verschiedenen Zeiten nach der Oestronbehandlung angegeben. Die Angaben in der ersten Kolonne beziehen sich auf die Messungen vor der Oestronzufuhr, die weiteren auf Messungen nach verschieden langer Behandlung. In den Rubriken vor dem  $O_2$ -Wert sind die Dosis und Dauer der Ovexbehandlung und das Körpergewicht des Tieres bei der Messung angeführt.

Auf den Tabellen III und IV ist ausserdem angegeben, zu welcher Zeit nach der Operation die  $O_2$ -Verbrauchbestimmung vorgenommen wurde.

Tabelle I enthält 7 *Normaltiere*, von denen 5 vor der Oestronbehandlung untersucht wurden. Die Tiere 3, 4, 5 sind dann 7 Tage, die Tiere 4 und 5 ausserdem nach 26 Tagen, und Nr. 4 50 Tage nach einer täglichen Ovexbehandlung mit 0.1 mg s.c. geprüft worden. Fall 6 und 7 wurden 29 und 51 Tage nach täglichen Oestroninjektionen von 0.5 mg gemessen.

In der Tabelle II finden sich 7 *hypophysenlose* Kaninchen. Fall 1 bis 3 sind unbehandelt, Fall 4 vor und nach 58-tägiger, Fall 5 nach 12- und 33-tägiger Oestronzufuhr und Fall 6 und 7 nach 2 bzw. 5-tägiger Behandlung untersucht worden.

Tab. III zeigt 4 *hypophysienstieldurchtrennte* Kaninchen, von denen Nr 1—3 33 Tage mit tgl. 0.1 mg Oestron behandelt wurden.

In der Tabelle IV endlich sind 3 unvollständig *stieldurchtrennte* Kaninchen angeführt, von denen Fall 1 und 2 nach  $2\frac{1}{2}$  und 3 Monaten untersucht wurden.

### Ergebnisse.

Die Ergebnisse sind im wesentlichen aus der Tabelle I—III ersichtlich. Zunächst sollen die vor der Oestronbehandlung erhaltenen besprochen werden.

**Tabelle I.**  
*Normale Kaninchen.*

Nach Behandlung mit Oestradiolmonobenzoat (Ovex, Leo, 0.001 g)												
Nr.	Vor der Behandlung				Ovex- behandlung				O <sub>2</sub> Ver- brauch		K. G.	O <sub>2</sub> Ver- brauch ccm/10 Min/kg
	K. G. g	O <sub>2</sub> Ver- brauch ccm/10 Min./kg	Dosis mg	Tage	K. G. g	O <sub>2</sub> Ver- brauch ccm/10 Min/kg	Dosis mg	Tage	K. G. g	O <sub>2</sub> Ver- brauch ccm/10 Min/kg		
1	1 700	73	—	—	—	—	—	—	—	—	—	—
2	2 800	76	—	—	—	—	—	—	—	—	—	—
3	2 150	77	0.1	7	2 050	85.3	—	—	—	—	—	—
4	2 400	95.6	0.1	7	2 500	94.6	0.1	26	2 800	69	—	—
5	2 400	89	0.1	7	2 200	88.5	0.1	26	2 500	68.25	2 750	60
6	—	—	—	—	—	—	0.5	29	3 200	44	—	—
7	—	—	—	—	—	—	0.5	29	3 200	49	3 250	61.5
							0.5	51			3 500	55

**Tabelle II.**  
*Hypophysevenlose Kaninchen.*

Nr.	Vor der Behandlung				Nach Behandlung mit Oestradiolmonobenzoat (Ovex, Leo)			
	K. G.	O <sub>2</sub> Verbrauch Mon. p. op. ccm/10 Min./kg	Overbehandlung Dosis mg Tage	K. G.	O <sub>2</sub> Verbrauch Mon. p. op. ccm/10 Min./kg	Overbehandlung Dosis mg Tage	K. G.	O <sub>2</sub> Verbrauch Mon. p. op. ccm/10 Min./kg
1	2 050	2 1/2 39	—	—	—	—	—	—
2	2 550	1 46.6	—	—	—	—	—	—
3	2 200	1 1/2 48	—	—	—	—	—	—
4	2 550	1 50	—	—	—	—	—	—
5	—	—	0.1 12	3 200	—	0.1 58	2 900	3 54
6	—	—	0.1 2	2 900	4 2	0.1 33	3 000	5 41
7	—	—	0.5 5	3 050	2 1/2 47	—	—	—

Tabelle III.

*Stieldurchtrennte Kaninchen.*

Nr.	Vor der Behandlung			Nach Behandlung mit Over, Leo				
	K.G. g	O <sub>2</sub> Verbrauch Mon. p. op.	ccm/10 Min/kg	Overbehandlung Dosis mg	Tage	K.G. g	O <sub>2</sub> Verbrauch Mon. p. op.	ccm/10 Min./kg
1	2 260	2½	55.5	0.1	33	2 100	4	55.6
2	2 750	2½	51	0.1	33	2 900	4	59.5
3	2 650	2½	42	0.1	33	2 300	4	55
4	2 300	2½	48	—	—	—	—	—

Tabelle IV.

*Unvollständig stieldurchtrennte Kaninchen.*

Nr.	K.G. O <sub>2</sub> Verbrauch			K.G.	O <sub>2</sub> Verbrauch		
	g	Mon. p. op.	ccm/10 Min/kg		g	Mon. p. op.	ccm/10 Min/kg
1	2 700	2½	78	2 970	3		72
2	2 350	2½	58	2 350	3		63.5
3	2 500	3	67.5	—	—		—

Der O<sub>2</sub>-Verbrauch der normalen Tiere variiert zwar zwischen 73 und 95,6 ccm/10 Min./kg, ist aber sowohl bei den hypophysenlosen wie bei den stieldurchtrennten so stark erniedrigt, dass der Unterschied deutlich hervortritt.

Bei der Berechnung der Unterschiede zwischen den Gruppen aus den Mittelwerten mit ihren mittleren Fehlern und dem mittleren Fehler der Differenz ergibt sich eine sichere *Senkung des O<sub>2</sub>-Verbrauches der hypophysenlosen* ( $M = 45,9 \pm 2,4$ ) *und der stieldurchtrennten* ( $M = 49,1 \pm 2,3$ ) *Tiere im Vergleich zu den normalen* ( $M = 82 \pm 4,3$ ), *aber kein Unterschied zwischen den hypophysenlosen und stieldurchtrennten.*

Der O<sub>2</sub>-Verbrauch ist also nach der Durchtrennung des Hypophysenstieles ungefähr ebenso stark herabgesetzt wie nach einer Hypophysektomie.

Die Werte der Tiere, bei denen die Stieloperation misslungen und der Hirnanhang ganz oder teilweise mit dem Infundibulum in Verbindung geblieben war, sind hier etwas höher als die der stieldurchtrennten. Nr. 1 hat einen O<sub>2</sub>-Verbrauch, der dem der normalen entspricht.

Betrachtet man nun die Ergebnisse nach der Oestronzufuhr, so scheint bei den Normaltieren der Tab. I allmählich eine Abnahme

des  $O_2$ -Verbrauches einzutreten. Besonders deutlich zeigt dies Fall 4, der nach 7-, 26- und 50-tägiger Behandlung mit täglich 0.1 mg Ovex Werte von 95, 94, 69 und 60 ccm/10 Min./kg aufweist.

Die Beurteilung des Resultates dieser Gruppe ist aber durch die schon oben erwähnte starke Streuung erschwert. Es wurden deshalb, da sich der Behandlungseffekt offenbar erst später als nach 7 Tagen zeigt, die Werte zu zwei Gruppen zusammengefasst. Die eine enthält alle Werte vor der Oestronzufuhr und die drei nach 7-tägiger Behandlung. Bei der anderen sind die übrigen 7, also nach 26—51 Tagen Behandlung vereinigt.

Vergleicht man nun diese beiden Gruppen miteinander und nimmt dabei an, dass jeder Wert von einem selbständigen Individuum stammt, so ergibt sich bei der ersten Gruppe der Mittelwert  $M = 84.9 \pm 3.0$  und bei der zweiten  $M = 58.1 \pm 3.6$ . Die Differenz beträgt  $26.8 \pm 4.7$  und  $D : \varepsilon_D = 5.7$ , d. h. es ist ein sicherer Unterschied vorhanden. Wegen der Korrelation der Werte wurde der Unterschied durch eine weitere Rechnung kontrolliert. Bei dieser wurde nur der Mittelwert der beiden Ergebnisse desselben Tieres berücksichtigt. Auch hierbei ergab sich eine sichere Differenz. Es kommt also bei den hier untersuchten normalen Kaninchen durch die Oestronbehandlung zu einer Abnahme des  $O_2$ -Verbrauches.

Eine derartige Berechnung erscheint bei den *hypophysenlosen* (Tab. II) und *stioldurchtrennten* Tieren (Tab. III) unnötig, da die Ergebnisse ohne weiteres erkennen lassen, dass die Oestronbehandlung den  $O_2$ -Verbrauch nicht verändert hatte.

### Diskussion.

Die starke Abnahme des  $O_2$ -Verbrauches nach der Hypophys-ektonie des Kaninchens stimmt mit früheren Befunden (PH. E. SMITH, 1927, u. a.) überein.

Als Ursache der ebenso grossen Senkung bei den stioldurchtrennten Tieren ist vielleicht ausser einer Hemmung der Produktion des thyreotropen Hormones noch eine besondere Stoffwechselstörung anzunehmen. Histologische Befunde an Schilddrüsen stioldurchtrennter Tiere weisen nämlich nicht konstant (WESTMAN, JACOBSON und OKKELS) oder nur nach besonderer Belastung (UOTILA) auf eine herabgesetzte Aktivität hin. Gegen eine Stoffwechselstörung infolge einer Hypothalamusschädigung sprechen die fast normalen Werte der unvollständig stioldurchtrennten

Kaninchen, bei denen das Operationstrauma dasselbe wie bei den stieldurchtrennten war. Dass die Werte verschieden und relativ niedrig sind, kommt offenbar durch die verschieden starke Verletzung des Hypophysenstieles zustande.

Die Verminderung des  $O_2$ -Verbrauches normaler Tiere nach langdauernder Behandlung mit Oestron stimmt mit den in der Einleitung erwähnten Befunden von GESSLER, GRUMBRECHT und LOESER und SHERWOOD überein und könnte durch eine Hemmung der Produktion des thyreotropen Hormons, wie sie HEYL, DE JONGH und KOOS (1934), GESSLER (1937) und GRUMBRECHT und LOESER (1938) z. B. annehmen, bedingt sein. Möglich wäre aber auch eine direkte Wirkung auf den Stoffwechsel, die SHERWOOD (1940) auf Grund von Versuchen an thyreoidektomierten, mit Oestradiol und Amniotin behandelten Kaninchen für wahrscheinlicher hält.

Der unveränderte  $O_2$ -Verbrauch der hypophysenlosen Kaninchen nach der Oestronzufuhr entspricht der Beobachtung von GESSLER (1937) an hypophysenlosen Ratten.

Wie in der Einleitung erwähnt, hatten WESTMAN und JACOBSON (1942) festgestellt, dass die Hypophyse stieldurchtrennter Kaninchen nach einer Oestronbehandlung wieder ihr normales Aussehen erhält. Aus der hier mitgeteilten Untersuchung des  $O_2$ -Verbrauches geht hervor, dass die Stoffwechselfunktion der Hypophyse durch eine derartige Behandlung nicht wiederhergestellt wird.

### Zusammenfassung.

Untersuchung des  $O_2$ -Verbrauches normaler, hypophysenloser und stieldurchtrennter Kaninchen vor und zu verschiedenen Zeiten nach einer Oestronbehandlung.

Der  $O_2$ -Verbrauch stieldurchtrennter Kaninchen ist ebenso stark herabgesetzt wie nach einer Hypophysektomie.

Durch die Oestronbehandlung sinkt der  $O_2$ -Verbrauch normaler Tiere, während er bei hypophysenlosen und stieldurchtrennten unverändert bleibt.

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## A Note on the Enzymic Hydrolysis of Certain Cholin Derivatives.

By

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In recent years a large amount of work has been done on the enzymic chemistry of acetyl cholin, on account of its great importance in nerve physiology. However, also other cholin derivatives have aroused the interest of biochemists. This is partly due to the application that some of these compounds have found in practical medicine, and partly because of the fact that by a simultaneous study of this whole complex of substances one may hope to make some progress in the understanding of the cholin esterase itself.

In the present note we are concerned only with one particular branch of this wide field, namely with the way in which the cholin esterase is influenced by eserine. Our problem may be expounded as follows (see AMMON 1940). It is well known that the enzymic hydrolysis of acetyl cholin is strongly retarded by eserine. This has been shown by AMMON and VOSS (1935) by injection of eserine in dogs, and by JONES and TODD (1935) for man. According to KAHANE and LEVY (1936) eserine also exerts in dogs a sensitizing influence on the blood pressure diminution by formyl cholin, acetyl cholin and propionyl cholin, *but not* by benzoyl cholin and acetyl- $\beta$ -methyl cholin, two derivatives which are rather slowly hydrolyzed by the enzyme. It is therefore an interesting question whether the hydrolysis of the latter two esters is

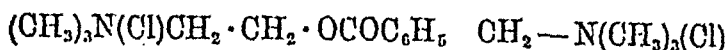
or is not retarded by eserine, or whether this influence of the alkaloid is only very weak. To quote literally the words of AMMON (1940, p. 397): "Es wäre wichtig, daher zu prüfen, ob das Eserin auch im Reagenzglas die Hydrolyse dieser beiden letztgenannten Ester gar nicht oder nur in wesentlich geringerem Ausmasse hemmt." The present author has searched the literature in vain for an experimental answer to this question, and it was therefore decided worth while to undertake an investigation of the kind indicated.

In order to follow quantitatively the progress of the hydrolysis we have chosen for reasons of simplicity and convenience the method of continuous titration, in which the hydrogen ion concentration of the reaction mixture is kept constant by continuously adding alkali and simultaneously controlling the pH by observing the colour changes of a suitably chosen indicator. The method is hence quite similar to that designed by KNAFFL-LENZ (1923) for the study of lipases. As regards the choice of indicator the following remarks may be made. STEDMAN c. s. (1932) originally used brom thymol blue, working at pH 7.4. However, when unpurified serum is used as a source of cholin esterase, difficulties were experienced due to the buffering action of the serum proteins. Later on, therefore, these authors used o-cresol phtalein, working at pH 8.6, but this was found to introduce additional difficulties due to the unstable nature of the substrates used, and hence this indicator was also abandoned. Finally, STEDMAN c. s. (1933) found cresol red to be a suitable indicator, working at pH 8.0. With this indicator it was possible to obtain well reproducible results, both the buffering action of the serum proteins and the non-enzymic hydrolysis being small, while the colour change of the indicator was well marked. The present author, after some initial work with brom thymol blue, therefore also decided to use cresol red. It is to be regretted that there is no investigation to be found in the literature of the influence of various indicators on the cholin esterase, analogous to the work of BAMANN and SCHMELLER (1931) on liver esterases. To avoid any possible inhibitory effect of the alcohol the indicator was used in an aqueous instead of in an alcoholic solution.

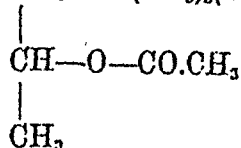
As enzymic solution we used serum from man. The subject of the purification of the cholin esterase is still in a very imperfect state and it was not considered advisable for the present purpose to make attempts at purification.



Of the two substances studied here, both were used in the form of their chlorides



Benzoyl cholin chloride



Acetyl- $\beta$ -methyl cholin chloride.

Benzoyl cholin chloride was synthesised by the method of FOURNEAU and PAGE (1914), which consists in heating the benzoic acid- $\beta$ -chloro-ethyl ester with trimethyl amin in benzene solution in a sealed tube. The acetyl- $\beta$ -methyl cholin chloride was kindly placed at our disposal by the A/S Ferrosan.

The measurements were carried out by dissolving 0.1 g of the substrate in 50 cm<sup>3</sup> of water, adding a few drops of the indicator (the same number of drops in all cases) and — usually —  $\frac{1}{2}$  cm<sup>3</sup> of serum, and, eventually, the eserine solution. The alkaloid was used in the form of its sulphate. The titrations were carried out with ca.  $n/100$  NaOH. In separate experiments the self-hydrolysis was determined and found to be unimportant. All the work was carried out at a temperature of  $22^\circ \text{C} \pm 0.1$ . The hydrolysis was usually followed for an hour, and the results were found to be reproducible to within around 5 %. Measurements were chiefly carried out with two alkaloid concentrations in the reaction mixture, namely

$$\begin{array}{l} \text{a) } 0.6 \cdot 10^{-4} \text{ g/cm}^3 \\ \text{and b) } 0.6 \cdot 10^{-4} \text{ g/cm}^3. \end{array}$$

Thus in a series of measurements with benzoyl cholin chloride the alkali consumption in a typical experiment was as follows

Without eserine	Case a	Case b
1.94 cm <sup>3</sup>	0.60 cm <sup>3</sup>	1.70 cm <sup>3</sup>
(The self-hydrolysis has been allowed for.)		

The retarding influence of eserine is evident from this table, and could easily be followed to much lower alkaloid concentrations. The results for acetyl- $\beta$ -methyl cholin chloride were quite similar,

and since they give nothing qualitatively new, we refrain from reproducing further figures.

To have an independent qualitative check on these results, some measurements were carried out in the WARBURG apparatus, using the well known gas analytic method developed by AMMON and RONA for the determination of esterase activity. The results were a qualitative confirmation of those already reported upon, and the numerical details and a closer description of these experiments may therefore be left out here.

In view of the results just reported we believe to have established the result that eserine also retards the hydrolysis of benzoyl cholin and acetyl- $\beta$ -methyl cholin by the cholin esterase of serum. As a matter of fact it seems to us that any other result would have been very difficult to understand on general theoretical grounds. However it must be admitted that as regards the interpretation of the experiments of KAHANE and LEVY the present work does not offer any new possibilities, except of course to warn against attempts at explanation based on the assumption that eserine does not influence the enzymic hydrolysis of the two esters studied here.

The author wishes to express his thanks to Prof. E. BIILMANN and Prof. R. EGE for their generous hospitality. He also wishes to thank Lektor Dr. S. VEIBEL for his interest in this work.

### Summary.

It is found that eserine retards the enzymic hydrolysis by serum cholin esterase of benzoyl cholin and acetyl- $\beta$ -methyl cholin. The relation of this result to certain experiments of KAHANE and LEVY is briefly discussed.

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Copenhagen.

## **Antithrombin Content of the Blood in Peptone Intoxication of Rabbits.**

By

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For the past 50 years, the influence of peptone poisoning on the animal organism has been an established fact, and during the same period a great number of widely differing scientific accounts have been published concerning this problem (*e. g.*, WÖHLISCH (1929)). The main interest has centred, however, on the antithrombin content of the blood, because it was recognized early (CONTEJEAN (1895)) that in dogs these conditions brought about a considerable increase in the amount of antithrombic substances in the blood. Several investigations into these problems established the liver as the principal site of the formation of these substances, and in recent years it has been demonstrated that in poisoning of this kind large amounts of heparin were poured out into the blood stream (WILANDER (1939)).

While these problems have been elucidated fairly well in dogs, the matter is still obscure in other animals — largely because the amount of thrombin-inhibiting substances given off to the blood stream is considerably smaller in other animals than in dog. Furthermore, with the analytical methods available so far for determination of the heparin content of the blood as well as its content of other thrombin-inhibiting substances, it has not been practicable to obtain any results that were even approximately accurate. So, it is no wonder that the findings recorded so far are highly confusing or even directly conflicting.

With the method described by ASTRUP and DARLING in 1942 for determination of the antithrombin content of the blood,

however, it has become possible to measure the presence of such substances accurately. Employing this new technique, I have reinvestigated these problems, especially in rabbits.

The animals here employed have been the ordinary commercial rabbits, chiefly white ones, weighing 2—3 kg.

The peptone used was Witte peptone, injected intravenously in varying amounts.

As the tolerance of rabbits for peptone was a rather unsettled question, some preliminary experiments were made with a view to this point.

Thus rabbits were found to be considerably more resistant to intravenous injection of peptone than are dogs. For instance, injection of 0.3 g. of peptone per kg. of body weight had no visible effect even in fasting rabbits, whereas this treatment in dogs produces almost invariably a pronounced state of shock.

For the production of shock in rabbits considerably larger doses were found to be required — most often up to 1—2 g. of peptone per kg. of body weight. Intravenous injection of such large amounts resulted most often in a pronounced shock with convulsions, loss of consciousness, involuntary passage of the urine and feces and, not infrequently, death of the animal.

In order to elucidate the effect of the peptone injections on the antithrombin content of the blood, some preliminary experiments were made in order to see whether the injection of small amounts of peptone might have an effect: 4 rabbits were given an injection of 2 cc. of a 5 % peptone solution, corresponding to about 0.05 g. per kg. Such an injection was found to have no influence whatever on the antithrombin content of the blood.

In some previous studies (VOLKERT 1941—42) it was demonstrated, however, that repeated injection of protein solutions — and also injection of solutions of high-molecular substances without any antigenic character — in a dose of about 0.05 g of the substance per kg. of the weight of the animal produced within two weeks a considerable increase in the antithrombin content of the blood. In these experiments the first two injections were found to have no effect whatever. But after 5—6 days the organism appeared to be sensitized and now each injection produced a characteristic brief rise in antithrombin, besides a slow general increase in antithrombin. It seemed obvious, therefore, to try whether this effect might be produced also by peptone and hence 4 animals were each given 5 injections of 0.05 g. of peptone per

kg. for 11 days. These injections were found to have no effect whatever; the organism was not sensitized to peptone. The increase of antithrombin on injection of peptone in dogs can therefore hardly be due to a sensitization of these animals through absorption of peptone from the intestinal canal. So the peptone shock appears not to be any kind of anaphylactic shock. This is confirmed by the fact that reinjection of peptone in rabbits 4 weeks after my attempt at sensitization proved to have just as little effect as the previous injections. On the other hand, reinjection of protein in amounts of 0.05 g. per kg. gave strong and characteristic antithrombin reactions.

In the next experiment 5 rabbits were given a larger amount of peptone — 0.3 g. per kg. — the very dose that gives a pronounced shock in dogs, together with a rise in antithrombin. As mentioned, this dose appears to have no influence whatever on the general condition of the rabbits, whereas it affects the antithrombin content of the blood, resulting in a pronounced fall in antithrombin — while in dogs the same treatment gives a marked rise in antithrombin. This fall makes its appearance immediately after the injection, the minimum antithrombin content being reached 5 minutes after the injection. The degree of this fall in antithrombin was extraordinarily uniform in all the cases, amounting to about 20 %. After this minimum was reached, the antithrombin content kept constant for a couple of hours, whereafter it commenced to rise again, reaching a normal level in 4—6 hours after the injection. These findings are presented graphically in Fig. 1.

In order to elucidate the cause of these peculiar findings, an experiment was made to see whether it might be possible also *in vitro* to lower the antithrombin content by addition of peptone. First, one drop of a 20 % peptone solution was added to 2 cc. of citrated *plasma*; this proved to have no effect whatever on the antithrombin content.

On the other hand, one drop of 20 % peptone solution added to 2 cc. of citrated *blood* gives a fall in the antithrombin content amounting to 20 %.

This finding reminds strikingly of the experiments reported earlier (VOLKERT 1942) in which the addition of India ink and chloroform to citrated blood likewise produced a fall in antithrombin content amounting to 20 %, whereas no such fall was seen on employment of citrated *plasma* instead of citrated *blood*.

As pointed out in detail in the report cited (VOLKERT, 1942), the fall in antithrombin content of the blood is due to injury to the red blood cells by the addition of India ink and chloroform, enabling these cells to combine with the antithrombin. So the influence of peptone on the antithrombin content of the blood is due most likely to the same mechanism and, as in the India ink and chloroform experiments, it is the variable antithrombin component of the blood that is eliminated on the addition of peptone — a component which, as described previously, is due to a heparin-like substance.

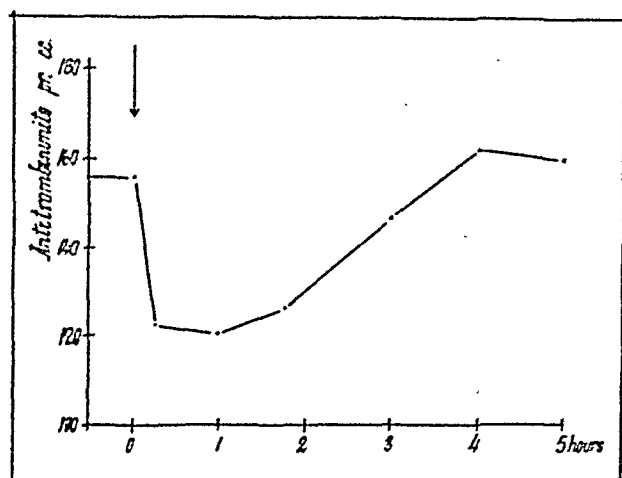


Fig. 1. The Antithrombin Content of the Blood by Injection of 0.3 g Witte Pepton per kg, at ↓.

Injection of larger amounts of peptone — sufficient to produce a pronounced shock — resulted in an altogether different antithrombin reaction. Thus, injection of 1—2 g. of peptone per kg. gave immediately a rapid rise in the antithrombin content of the blood amounting to about 25—30 %. This was followed by a gradual fall, the antithrombin concentration reaching a normal level in about 2 hours. But, instead of staying at this level, the fall continued, reaching a minimum in about 2 additional hours. In all 5 animals this minimum was represented by a fall of about 20 %.

The antithrombin content did not keep constant at this level, however, but soon commenced to rise again, reaching a normal level in about 4 hours — and then it stayed at that level. These features are presented graphically in Fig. 2.

These experiments show that it is possible also in rabbits by

intravenous injection of peptone to produce a rise in the antithrombin content of the blood if only the dose is sufficient to produce shock.

In previous experiments on rabbits in which it has been possible to produce a rise in the antithrombin content of the blood (by immunization, anaphylactic shock and obstructive jaundice), it has further been possible completely to block this effect by 5 injections of 0.1 cc. of India ink per kg. An experiment was carried out, therefore, to see whether such blocking would hold

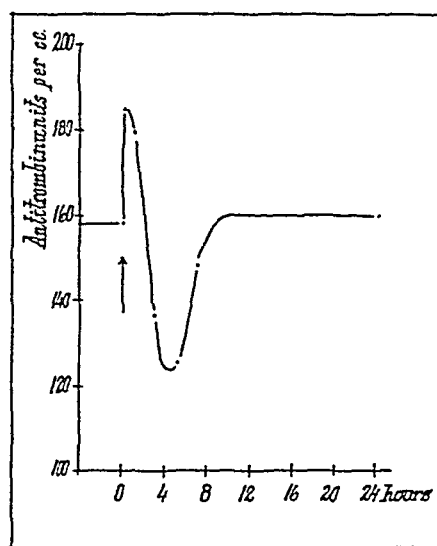


Fig. 2. The Antithrombin Content of the Blood by Injection of 1.5 g Witte Pepton per kg, at ↑.

good also on injection of peptone — and this was found to be the case.

In 3 animals in which an India ink blockade was established beforehand, it was not possible to produce any rise in the antithrombin content even by injection of maximal doses of peptone. This observation, I think, suggests very strongly that it is the same substance that is produced in the peptone poisoning as is found during immunization, anaphylactic shock and obstructive jaundice, involving an increase in the variable antithrombin component of the blood — *i. e.*, that a heparin-like substance is produced and poured into the blood stream. That the rise in antithrombin observed in peptone intoxication is only about one-half of the rise observed in the other conditions may possibly



be due to injury to the red blood cells associated with peptone intoxication, binding in this manner a part of the antithrombin formed.

Correlating the experimental results concerning the antithrombin content of the blood in peptone intoxication in rabbits the interpretation appears to be probably as follows:

1. The small amounts of peptone — 0.05 g. per kg. — are too small to affect the blood corpuscles to such an extent as to produce a fall in antithrombin. Nor can such an amount of peptone have a stimulating effect on the organs which give off the heparin-like substances and thus produce a rise in antithrombin.

2. The moderate doses of peptone — 0.3 g. per kg. — affect the blood corpuscles and give rise to a fall in the antithrombin content of 20 %. This means that all the antithrombin is removed which normally results from the heparin-like substances present in the blood. This amount of peptone is still too small, however, to act as a stimulant and produce an increase in antithrombin.

3. Injection of peptone in amounts as large as 1—2 g. per kg., which produces shock, gives rise to stimulation of the system in which the heparin-like substances are formed, so that these substances are now poured out into the blood stream in an increased amount. At the same time, these large amounts of peptone have a strong effect upon the blood corpuscles, so that the blood cells combine with some of the antithrombin formed, partly masking the total antithrombin production. Furthermore, this effect on the blood corpuscles appears to be rather protracted. This is evident from the fact that the fall in antithrombin content subsequent to the sudden, but probably brief, production of heparin-like substances does not stop at the normal level, but keeps on till it reaches a value of about 20 % below this level. When this stage is reached, not only the antithrombin produced by the experimental conditions is removed from the blood, but also that amount of the normal antithrombin that is due to the heparinlike substance.

The effect of the peptone injection on the blood corpuscles lasts for about 4—5 hours; then the antithrombin content of the blood rises again to a normal level.

Thus it has been demonstrated that an increase in the amount of heparin-like substances in the blood can be produced in rab-

bits by peptone intoxication — just as in dogs and cats — but that in rabbits it is absolutely necessary for this purpose to produce a shock. As to the reaction of thrombin-inhibiting substances in rabbits on injection of small amounts of peptone, it may be that with a suitable dosage, similar phenomena may be produced in dogs and cats.

### Summary.

1. Experimental studies are reported on the tolerance and reaction of rabbits to intravenous injection of Witte peptone, and it is illustrated that shock can be produced also in rabbits if only the peptone is injected in sufficient amount.

2. The antithrombin content of rabbit blood is examined after injection of varying amounts of peptone. It is pointed out that, when shock is produced, it is possible also in rabbits to elicit an increased formation of heparin-like substances in the blood. In addition, it is demonstrated that administration of smaller amounts of peptone may give a decrease in the antithrombin content of the blood. These findings are discussed.

The studies here reported were carried out with the aid of a grant from "Løvens kemiske Fabriks Legat til Minde om Apoteker Aug. Kongsted". The considerable amounts of thrombin required for the determination were generously placed at my disposal by Løvens kemiske Fabrik, Copenhagen

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## **Antagonism between Bile Acids and Substances Stimulating Smooth Muscle, Especially Histamine.**

By

NILS EMMELIN.

(Received 20 February 1943.)

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In previous experiments (1941) we have found that the sodium-salts of glyco- and taurocholic acids considerably decrease the sensitivity of isolated small intestine of guinea-pig to histamine (Hi). In this paper a more detailed investigation is made into the effect of the bile acids on the sensitivity to Hi of smooth muscle. Beside Hi, acetylcholine and EULER-GADDUM's P-substance have also been investigated. Sodium glycocholate and sodium taurocholate (MERCK) were used in the experiments.

### **Experimental.**

#### **A. Histamine.**

*Experiments on small intestine of cat.* The experiments were carried out on cats under chloralose. The belly was opened medially, and a rubber balloon introduced into the gut by means of an incision in a section of the jejunum with retained circulation. The balloon was filled with water at body temperature, and attached by means of a rubber tube to a registering water-manometer. The gut and the abdominal wall were closed round the rubber tube. Hi was injected at constant rate into a femoral vein. Bile-acid solution was injected in the other femoral vein.

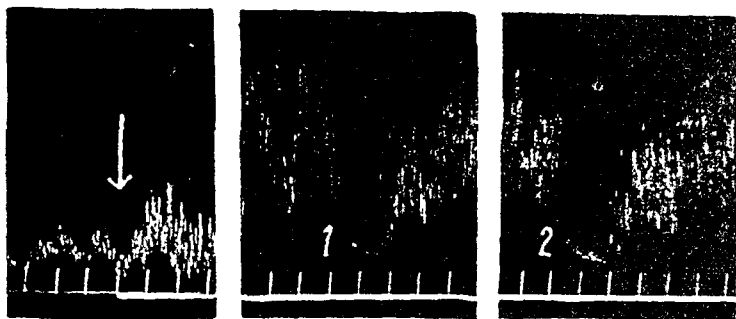


Fig. 1. Record of jejunal tone and motility (cat, 2.9 kg). At ↓ slow injection of Hi (1  $\gamma$ /kg and minute) continuing during the whole course of the experiment. At 1 sodium glycocholate (5 mg/kg) was injected and at 2 the same dose after section of the vagi in the neck, the splanchnic nerves and ligature of the suprarenal vessels. Between the sections of the tracing there is an interval of 10 minutes. Time-marks in minutes.

Fig. 1 represents an experiment of this kind. During injection of Hi the motility and tone of the gut were considerably increased. The graph shows that injection of bile acid caused a transient decrease of both motility and tone. The effect remains after section of the vagi in the neck and the splanchnic nerves, and ligation of the suprarenal vessels.

In experiments on isolated small intestine suspended in Tyrode-solution, the addition of bile acid to the bath also decreased sensitivity to Hi, this too indicating that the effect is peripheral.

In experiments on the whole animal, the least dose of Na-glycocholate and taurocholate that had effect was about 2 mg per kg body weight.

*Isolated small intestine of guinea-pig.* Pieces of guinea-pig ileum were suspended in Tyrode-solution in the usual way. The first part of fig. 2 shows an experiment with taurocholate. The bile salt considerably decreased sensitivity to Hi. After adding taurocholate up to a concentration of 1:40 000, the same Hi-dose caused a contraction no greater than the one resulting from a third of the dose before the addition of bile acid. In a concentration of 1:10 000 the bile salt rendered the Hi-dose completely ineffective.

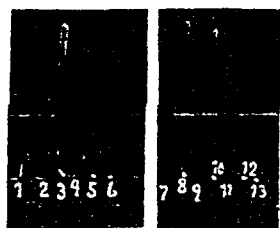


Fig. 2. Guinea-pig's small intestine suspended in 2 cc Tyrode-solution. 0.005  $\gamma$  Hi was added at 1. 0.015  $\gamma$  Hi at 2, 4 and 6. At 3 the bath contains sodium taurocholate 1:40 000, and 1:10 000 at 5. Before 7 the gut was washed with Tyrode. 0.015  $\gamma$  Hi was added at 7, 9, 11, and 13. At 8 the bath contains sodium dehydrocholate 1:40 000, 1:4 000 at 10 and 1:1 000 at 12. Time in minutes.

The anti-histamine effect of taurocholate is still apparent in a concentration of 1 : 100 000. The least concentration of glycocholate that has effect is about 1 : 60 000. The effect is noticeable within the first half-minute after adding bile acid, and is maximal after about three minutes. On washing the gut with fresh Tyrode, sensitivity soon reappeared.

*Isolated guinea-pig uterus.* It was shown by the same method that isolated uterus of guinea-pig also loses its sensitivity to Hi after preliminary treatment with the bile salts.

*Blood-pressure of cat.* The blood-pressure in the femoral artery was registered under chloralose with a mercury-manometer. Hi was injected into a femoral vein before and during slow injection of bile salts. 2—5 mg of glycocholate or taurocholate per kg body weight and minute caused a slight fall in blood-pressure. After infusion of bile acid had begun, Hi (0.3—5γ/kg) caused a rather smaller fall in blood-pressure. The effect of Hi in decreasing blood-pressure, is however, never completely abolished by the bile salts.

*Do bile acids interfere with the biological assay of Hi?* The isolated intestine and uterus of guinea-pig and the blood-pressure of cat are generally used for quantitative determination of Hi. In connexion with the observations described here, the question arises whether the occurrence of bile acids in body fluids, e. g. blood, bile, intestinal content, may interfere with the assay of Hi.

The blood content of bile acids is not known for certain. Some authors (e. g. ROSENTHAL and WISLICKI 1926, JENKE 1939) deny that bile acids occur normally, while others give a normal concentration of bile acid of c. 1 : 20 000 (GREENE and ALDRICH 1927, COQUELET 1927, LIGHTMAN 1938). Considerably higher concentrations have also been given as normal. In jaundice due to stasis, it is said that the concentration may rise to 1 : 5 000 or more (GILBERT, CHABROL and BÉNARD 1920, MINIBECK 1938, GIGON and NOVERRAZ 1940).

If bile acids can occur in these high concentrations in the blood it does not seem out of the question that they may interfere with the assay of Hi. In determining the Hi-content of liver-extracts, which is essential because the liver plays an important part in liberating Hi during anaphylaxis, the amount of bile acids in the liver may also be a disturbing factor.

The following experiments were made. Samples containing Hi (15—50γ/litre) and glyco- or taurocholate (1 : 20 000—1 : 5 000)

in Tyrodesolution were tested on guinea-pig ileum, partly direct, partly after extracting according to CODE (1937). A definite interference of the bile acids could not be shown in any experiment, although it could be calculated that a concentration antagonistic to Hi was reached in the bath. The explanation probably is that Hi causes contraction of the gut so swiftly that the bile acids do not have time to exert an antagonistic effect.

*Using Hi-antagonism in biological testing.* Fig. 3 is an example that the antagonism of the bile acids can be used to demonstrate the presence of substances whose activity is concealed by the simultaneous presence of Hi. The occurrence of Hi in gastric juice has been shown by MACINTOSH (1938). BLOCH and NECHELLES (1938) have found that gastric juice from dogs and man contains a toxic substance whose chemical nature is unknown, and which relaxes isolated small intestine of guinea-pig. On testing gastric juice from dogs with Pavlov pouch, we have found in some instances that after preliminary treatment of the gut with bile acids the juice has a relaxing effect on intestine of guinea-pig, contrary to the contracting effect that would be observed due to the presence of Hi in the juice. Fig. 3 shows an experiment of this kind.

*The effects of bile acids on symptoms probably caused by liberated Hi.*

1. *Snake venom.* Investigations of FELDBERG and KELLAWAY (1937), FELDBERG, HOLDEN and KELLAWAY (1938) and others have shown that various snake venoms form lysolecithin, which liberates Hi, from lecithin in the tissues. A number of the effects of snake venoms can be accounted for by Hi thus liberated. Snake venoms cause among other things a contraction of the guinea-pig's small intestine.

In our experiments we investigated whether bile acids affect this contraction. Fig. 4 shows an experiment of this kind. Venom of *Vipera berus* was used. Since, as appears from the figure, the snake venom causes a marked tachyphylaxy (desensitization), the experiment was carried out on two different pieces of gut. The figure shows that sodium glycocholate in a concentration of 1:10 000 completely abolishes the effect of the snake venom.

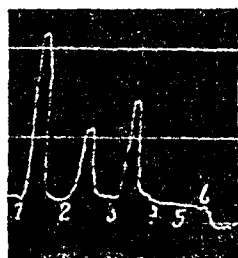


Fig. 3. Atropinized guinea-pig gut suspended as in fig. 2. 0.02  $\gamma$  Hi was added at 1, 3, and 5. At 2 and 6, 0.4 cc neutralized gastric juice from a sham-fed dog with oesophageal fistula and Pavlov pouch, at 4, sodium glycocholate 1:5 000. Time in minutes.

It will be seen that after the sensitivity to Hi has been re-established by washing (at 9), the snake venom causes (at 11) only a slight contraction (similar to that at 3). A reasonable explanation of this may well be that the bile acids do not inhibit the liberation of Hi, but prevent the liberated Hi from causing a contraction (at 8). Although the gut does not contract, desensitization occurs — e. g. because the greater part of the lecithin or Hi available for the reaction was liberated when the snake venom was added for the first time (at 8).

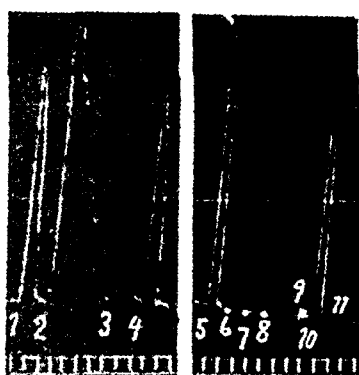


Fig. 4. Isolated guinea-pig gut. Two pieces have been used, one for each part of the graph. At 1, 4, 5, 7, 10, 0.02  $\gamma$  Hi was added, at 2, 3, 8, 11, 0.2 mg snake-venom. At 6, Na-glycocholate 1:10 000. At 9, washing and fresh Tyrode-solution. Time in minutes.

2. *Trypsin*. ROCHA e SILVA (1940) has found that in its proteolytic activity trypsin liberates Hi from the tissues, and that i. a. the intestine of guinea-pig is contracted by trypsin. In our experiments we found that bile acids counteract these contractions caused by trypsin.

3. *Dale-Schultz's reaction*. Liberation of Hi in anaphylactic shock has been described by a number of authors. It is well known that, added to the isolated gut of a sensitized guinea-pig, antigen causes a contraction (SCHULTZ 1910, DALE 1913).

The effect of the bile acids on Dale-Schultz's reaction was investigated. Guinea-pigs were sensitized in the usual way by intraperitoneal injection of ovalbumin or horse-serum. Two to three weeks later experiments were carried out on isolated intestine. After preliminary treatment with bile acids, antigen caused no contraction of the gut.

*Chloride of mercury*. In the coagulation of albumen caused by mercury salts, a liberation of Hi is considered to occur. In perfusion of the gut or liver with a solution containing mercuric chloride, Hi therefore occurs in the perfusate (HEUBNER and BACHMANN 1937, FELDBERG and KELLAWAY 1938).

In agreement with this we find that  $\text{HgCl}_2$  (in conc. 1:40 000) produced a contraction of the isolated intestine of guinea-pig. On adding bile acid,  $\text{HgCl}_2$  causes no contraction.

*The active component in the bile-acid molecule*. Certain amino acids — histidin, arginin, cystein — decrease the Hi-sensitivity

of the guinea-pig gut (v. ACKERMANN and WASMUTH 1939). The assumption lies to hand that the anti-Hi effect of the glyco- and taurocholic acids is due to the fact that the amino acid glykokoll and taurin, which closely resembles cystein, are components of the molecule of bile acids. But experiments on guinea-pig ileum with glykokoll and taurin showed that even in very high concentrations (1:200), these substances do not perceptibly diminish the sensitivity of the gut to Hi. Experiments on guinea-pig ileum with simple bile acids showed that the anti-Hi effect is exerted by the simple component of bile acid. Sodium desoxycholate (B. D. H.) and dehydrocholate (sodium salt of acidum dehydrocholicum Leo) have been investigated. The least concentration of desoxycholate that had effect was about the same as that of taurocholate, i. e. 1:100 000. Dehydrocholate was the least active of the bile salts investigated, not being active in conc. smaller than 1:7 000. Fig. 2 shows that dehydrocholate is much less effective than taurocholate.

*Effect of saponines.* It is to be noted that if the four bile acids investigated are compared for their anti-Hi effect, the same relation is found between them as for the most prominent physical quality, the decrease of surface-tension. The idea immediately occurs that the change in the muscle-cells brought about by the bile acids and expressed in a lowered sensitivity to Hi has to do in some way with the surface-activity of the bile acids. To obtain some idea of the significance of the surface-activity, it was investigated whether other strongly surface-active substances have an anti-Hi effect. Experiments were made with saponines. A saponine that haemolyzed human blood even in a concentration of 1:70 000 showed, when investigated in concentrations of 1:100 000—1:15 000, no antagonism to Hi.

In a concentration of 1:10 000 the saponine itself caused a contraction of the gut. This resembled the one obtained on adding snake venom to gut or antigen to sensitized gut. Desensitization was also demonstrated. It may be that in his high concentration the saponine inflicts on the cells a lesion that results in liberation of Hi.

### B. Acetylcholine.

The effect of glyco- and taurocholate on the sensitivity of isolated intestine of guinea-pig to acetylcholine was investigated. In a concentration of 1:50 000 these bile acids clearly diminished



the stimulating effect of acetylcholine (0.3  $\gamma$  acetylcholine hydrochloride in a bath of 2 cc). In the same concentration they diminished the motor effect on the gut of carbaminoylcholine (0.05  $\gamma$  "Doryl Merck" in 2 cc bath).

### C. Substance P.

In extracts of gut and brain, v. EULER and GADDUM (1931) demonstrated a factor, called substance P, stimulating the motor activity of the intestine. We investigated the effect of bile acids on the sensitivity to this substance of the isolated intestine of guinea-pig. In these experiments 0.5—2 units of P-substance caused a strong contraction of the gut (in a bath with 2 cc Tyrode-solution). In a concentration of 1:20 000, glyco- or taurocholate rendered these doses of P-substance quite ineffective.

### Discussion.

The experiments show that glyco- and taurocholic acids counteract the stimulating effect of Hi, acetylcholine and P-substance on smooth muscle. It is not apparent from the experiments whether this antagonism between substances occurring in the body has physiological importance, but it should have methodological interest.

OIDE (1920) showed that extracts of lung and the mucous membrane of the intestine cause contraction of smooth muscle and fall in blood-pressure, and that these effects are weakened by bile or bile salts. The experiments described here seem to make it likely that the active factor in the extracts was Hi, which can be extracted from these tissues in large quantities.

The antagonism we have found may perhaps be made use of in demonstrating substances relaxing the intestine in extracts also containing Hi, acetylcholine, or substance P.

The effects of the bile acids described here resemble the anti-histamine and anti-acetylcholine effects of the xanthines described by EMMELIN et al. (1941). Like the xanthines, the bile acids only partially antagonize the blood-pressure effects of histamine.

In connexion with the capacity of the bile acids to decrease the effect of snake venom and anaphylactic reaction on smooth muscle it may be of interest to point out that it has been shown

that snake venom has a less toxic effect after the addition of bile, and that many authors have ascribed the bile acids a therapeutic importance in various anaphylactic conditions (v. COTTET 1939).

### Summary.

In antagonizing the action of histamine and in suppressing anaphylactic symptoms the bile acids have properties similar to those previously described for the xanthines. The bile acids render smooth muscle insensitive to histamine; they also antagonize the motor action of acetylcholine and of substance P on smooth muscle. The effect of histamine on blood-pressure is only partially antagonized by bile acids.

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## **The Reticulocyte-Ripening Index after Loading with Liver Extract and Tyrosine.**

By

**CLAUS MUNK PLUM and RUTH PLUM.**

(Received 28 February 1943.)

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In an earlier report one of us (C. M. P., 1942) showed that the ripening of reticulocytes into mature red blood corpuscles could be accelerated by an as yet unknown substance. This reticulocyte-ripening substance is found in plasma and in various organs such as spleen, liver and bone marrow.

The chemical nature of the substance is still unknown but JACOBSEN and PLUM (1942) have shown that the substance met with in liver consists of at least two factors, one thermostable and one thermolabile; the former being identified as tyrosine.

When to the liver extract, which itself contains some tyrosine, a further quantity of tyrosine is added, a greater increase is obtained in the activity of the reticulocyte-ripening substance than that produced by the added tyrosine itself. The effect of the reticulocyte-ripening substance found in plasma cannot usually be increased by adding tyrosine. Since under normal conditions the quantity of tyrosine in plasma is small, the question arose as to how far the substances found in plasma and liver extract respectively were chemically different.

JACOBSEN and PLUM (1942) have shown that the reticulocyte-ripening substance found in plasma may probably be regarded as a coupling between tyrosine or a tyrosine-like substance and an as yet unknown thermolabile factor. After a total blockade of the reticulo-endothelial system, a large fall in the quantity of reticulocyte-ripening substance was found, and this slowly rose again when the blockade was lifted. At the same time it appeared

that by adding tyrosine to plasma from the animals treated, we could obtain normal activity from the plasma. This gave us the basis for the assumption that the coupling between tyrosine and the reticulocyte-ripening substance occurs in the reticulo-endothelial system.

These results made us to test whether the organism is able to transform substances which can be activated by tyrosine into substances that are no longer capable of being activated.

In the present paper an account will be given of some investigation concerning this point; as substances given to the animals we used liver extract, tyrosine and saline.

Liver extract itself is active, but by addition of tyrosine the effect of the reticulocyte-ripening substances is increased. Tyrosine itself is only slightly active and after adding saline to the blood corpuscles the reticulocytes ripen very slowly (C. M. P., 1942).

We gave all the substances by intramuscular injections to rabbits, blood samples were taken from time to time and the investigations were made on the plasma as earlier described (C. M. P., 1942).

The ripening index was determined on plasma alone as well as on plasma with addition of tyrosine. In this way we determined the amount of ripening substances and the amount of ripening substances + the amount of tyrosine activated substances. This shows whether the injected substances in liver extract are transformed into substances not able to be activated by tyrosine or remain as injected.

We used saline as a control and tyrosine was given to test whether the tyrosine content in liver extract is able to liberate some reticulocyte-ripening substances in the organism or not.

### Method.

White buck rabbits of 2,500 grammes were used for the experiments; their food before and during the experiments consisted of turnips and cabbage leaves.

Blood samples were taken with the apparatus mentioned by Sjöwall (1936). For each determination 8 c. c. blood was taken and stabilized with 2 c. c. sodium citrate (3.8 %). The determinations of the ripening index in plasma were made according to the method described by C. M. Plum (1942). The plasma was tested both with and without the addition of tyrosine.

### Results.

*Loading with liver extract.* To each rabbit used for the experiments was given an injection of 10 c. c. Hepsol fortior »MCO» in the muscles of the gluteal region, 5 c. c. in each side.

Tabelle 1.

*The results of the intramuscular loading with some substances, active or inactive with regard to the reticulocyte-ripening in vitro. The results are given as average of five determinations for each substance.*

Experi- mental time in hours	Injections of Hopsol fortior 10 c. c.				Injections of saline 10 c. c.				Injections of Tyrosine 0.3 % 10 c. c.			
	1.9 c. c. Plasma + 0.1 c. c. NaCl 0.9 %		1.9 c. c. Plasma + 0.1 c. c. Tyrosine 1 %		1.9 c. c. Plasma + 0.1 c. c. NaCl 0.9 %		1.9 c. c. Plasma + 0.1 c. c. Tyrosine 1 %		1.9 c. c. Plasma + 0.1 c. c. NaCl 0.9 %		1.9 c. c. Plasma + 0.1 c. c. Tyrosine 1 %	
	Ripening Index				Ripening Index				Ripening Index			
	Average	Mean error of average $\frac{m}{\sqrt{N}}$	Average	Mean error of average $\frac{m}{\sqrt{N}}$	Average	Mean error of average $\frac{m}{\sqrt{N}}$	Average	Mean error of average $\frac{m}{\sqrt{N}}$	Average	Mean error of average $\frac{m}{\sqrt{N}}$	Average	Mean error of average $\frac{m}{\sqrt{N}}$
0	0.75	0.02	0.78	0.03	0.72	0.02	0.76	0.02	0.73	0.02	0.77	0.02
2½	0.81	0.04	1.30	0.10	0.74	0.02	0.78	0.03	0.76	0.02	0.80	0.03
5	0.90	0.04	1.02	0.07	0.73	0.03	0.78	0.04	0.84	0.03	0.86	0.02
23	0.98	0.02	0.94	0.04	0.75	0.02	0.79	0.02	0.88	0.02	0.90	0.03
30	0.89	0.03	0.90	0.04	0.76	0.02	0.80	0.03	0.83	0.04	0.85	0.03
47	0.88	0.02	0.85	0.02	0.75	0.04	0.80	0.04	0.80	0.02	0.84	0.03
71	0.78	0.02	0.81	0.03	0.76	0.03	0.72	0.03	0.78	0.03	0.80	0.02
95	0.78	0.02	0.80	0.02	0.78	0.03	0.81	0.02	0.78	0.02	0.79	0.03

As it will be seen from the table, the ripening index in plasma rises steadily during the first 24 hours and then falls again. When a slightly increased ripening index is found after the lapse of 3—4 days, it must be ascribed rather to the effect of the blood letting (PLUM, 1943) than to the effect of the dose of Hepsol fortior.

By adding tyrosine (0.1 c. c. 1  $\frac{1}{100}$  tyrosine) to the plasma samples we find that a short time (2 $\frac{1}{2}$  hours) after the injections the plasma can be activated and that this property then declines in the course of about 24 hours. When maximum of directly determined ripening index is reached 24 hours after the injection, no further activation of tyrosine can be obtained.

These results indicate that the principle in injected liver extracts can be transformed into substances of the same character as normally found in plasma.

*Loading with saline.* As a control 10 c. c. physiological saline was injected intramuscularly just as in the above mentioned experiment. Thereby a steady rise occurred in the ripening substances in the plasma and this was maintained throughout the entire period of the experiment. This rise can hardly be due to the injection; for, as previously shown, we find a steady rise in the quantity of ripening substances in the plasma when an animal is anemized by blood letting (PLUM, 1943) and this effect was indeed unavoidable by reason of the fairly large quantity of blood that is used for each count. On activating with tyrosine we see only a slight rise in the ripening index, a rise which does not exceed that found for normal plasma and must be attributed to the effect of the tyrosine itself. Consequently the injection of physiological salt solution shows that the quantity of fluid administered is in no way capable of mobilising the reticulocyte-ripening substance in the organism.

*Loading with tyrosine.* Previous investigations by JACOBSON and SUBBAROW (1935) among others have shown that tyrosine is to be found, even if only in small quantities, in liver extract. Investigations made in this laboratory have shown that in the Hepsol fortior used in these experiments, there was 0.3 % tyrosine.

We therefore injected 10 c. c. 0.3 % tyrosine solution into the muscles and took blood samples as usual. The results can be seen from the table.

Here, just as with the liver extract injections, we find a rise in the content of ripening substances in the plasma for the first

24 hours after which a fall sets in. The rise is not however quite so high as after liver extract.

On adding tyrosine to the plasma sample no increase apart from what must be attributed to the effect of the tyrosine itself is found. It must thus be concluded either that the administering of tyrosine causes a mobilisation of ripening substances already found in the organism or that tyrosine can be transformed in some way to an active principle. This shows that a part, if not the total increase found after injections of liver extract must be due to the tyrosine content of the latter.

All these investigations showed that the substances in liver extract activated by adding tyrosine *in vitro*, are also in the normal organism transformed to active substances and that the effect of liver extract exceeds that due to its tyrosine content.

### Summary.

After injections of liver extract in rabbits, an increase in reticulocyte-ripening substances in plasma is found. In the first 24 hours after the injection the ripening substances in plasma can be activated with tyrosine in contrast to normal plasma and are therefore taken to be the unaltered liver substances.

Injections of tyrosine alone in the same quantities as found in the liver extract used, also produced an increase, but exclusively of fully activated ripening substances. Controls with saline give no rise.

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ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 5, SUPPLEMENTUM XIV

*From the Chemical institution, Karolinska  
Institutet, Stockholm*

ON THE  
HISTO- AND CYTOCHEMICAL  
DETERMINATION OF  
PHOSPHORUS

BY

BO NORBERG

STOCKHOLM 1942



## Corrections

*to Acta physiologica scandinavica, vol. 5, Supplementum XIV:*

**On the histo- and cytochemical determination of phosphorus.**

On page 5, line 14 from the foot of the page, insert 111 after 89 in the parenthesis.

- • 50, line 18, insert method before more.
- • 56, Table 27, head of first column, read P in  $10^{-3}\gamma$  for P in  $10^3\gamma$ .
- • 60, line 11, read 0.35 mm for 35 mm.
- • 76, • 2, from the foot of the page, read 0,208 % for 2,08 %.
- • 78, Table 34, line 6, insert ( $I_0 = 400$ ) after Sector-value.
- • 79, • 35, head of third and fifth column, read Exocrine part for Endocrine part.
- • 83, line 7, read could not for cannot.

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## CHAPTER 1.

### Introduction.

Modern histo- and cyto-chemistry already disposes over a number of very sensitive methods for the demonstration and determination of certain substances in cell-groups, single cells and even parts of cells. A *qualitative* determination of the distribution of certain substances in the cell can be obtained by an examination of the distribution of the ash — spodography (see e. g. BAGINSKI, 7, UBER, 122). The spodography may be supplemented by several sensitive qualitative chemical reactions — spot tests (see FEIGL, 54). *Quantitative* analyses on a histo- and cyto-chemical scale, however, can be carried out only for a couple of groups of substances, namely, enzymes and substances with a strong natural light-absorption.

For the quantitative determination of *enzymes* a series of methods have been developed by LINDERSTRØM-LANG and co-workers (6, 60, 66, 88; 89). By means of a skilful adaptation to a suitable micro-scale and since *one* enzyme molecule can give rise to *several* of the radicals (e. g.  $-\text{COOH}$ ) that are being determined, titrimetric methods can be employed right down to cell-dimensions. The determinable quantities are in the vicinity of  $10^{-1}\gamma$ ,<sup>1</sup> calculated as nitrogen with a minimum error of about  $5 \cdot 10^{-3}\gamma$ . To increase the sensitivity considerably above that already achieved seems, with titrimetric methods, to offer insuperable difficulties.

With the aid of two physico-chemical methods LINDERSTRØM-LANG has succeeded in extending the given measuring scale downwards to the power of ten. The methods he employed are a gasometric method based upon the Cartesian diver and a method that depends upon the change in the specific weight under the influence of enzymes (80, 81, 83—86, 125, 129).

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<sup>1</sup> The symbol  $\gamma$  is here and in the following pages used for microgram (one millionth of a gram).

CASPERSSON and his co-workers (15—43, 59, 78, 113) have exploited the strong natural light-absorption of certain substances, especially in the ultra-violet wave-length region (*nucleic acids, protein substances*), for the working out of quantitative methods for the analysis of cell details; and they are able to determine with great accuracy amounts of nucleic acid of the order of magnitude  $10^{-8}\gamma$ . Even in visible light one can get down to extraordinarily small amounts of substance ( $10^{-7}\gamma$  hematoporphyrin, 33).

From the above brief summary it will be seen that the methods that are best adapted for the cyto- and histo-chemical scale are physico-chemical. Of the physico-chemical methods that are used in biochemistry the photometric are probably among the most many-sided. In the light of the excellent results obtained with micro-photometry by CASPERSSON and his co-workers one felt justified in trying to work out a technique involving the use of known colour-reactions on histological and cytological material, which would open up many new spheres of research for quantitative biochemical investigations.

As it was from the outset clear that considerable technical difficulties must be overcome before a micro-technique with the desired sensitiveness could be achieved, and as at the beginning there was no means of telling which colour-reaction might first be applied on the intended micro-scale, I deemed it most suitable to limit my task to the working out of a micro-photometric technique and to the testing of the same on appropriate biological material. In part I I have described the working out of a method for determining phosphorus for histo- and cyto-chemical analyses; and in part II have been given some of the results gained with this method on different material.

## PART I.

# The working out of a method for the histo- and cyto-chemical determination of phosphorus.

### CHAPTER 2.

#### Principles and theoretical approach.

The order of magnitude of the amounts of substance occurring in a single cell is of course very various. As regards nucleic acid or protein, that are always present in comparatively large quantities, the amount is probably from about one to about ten per cent of the weight. If the specific weight of the cell is assumed to be  $= 1$ , then a mammalian cell regarded as a sphere with a radius of  $5\mu$  will weigh  $5.2 \cdot 10^{-4}\gamma$ . With a nucleic acid content of 10 % the weight of the nucleic acid will be  $5 \cdot 10^{-5}\gamma$ , and the nucleic acid phosphorus will weigh about  $3.5 \cdot 10^{-6}\gamma$  (62). A cell with ten times the above radius would under the same conditions contain approximately  $3.5 \cdot 10^{-3}\gamma$  nucleic acid phosphorus. For a nitrogen-content of 12 % in nucleic acid (62) the nucleic acid-N would in the latter case be about  $6 \cdot 10^{-3}\gamma$ . It is evident that for cyto-chemical investigations we need micro-methods that allow of determinations of about *a thousandth part of a microgram and smaller amounts*. Analyses in this order of magnitude will in the following be referred to as the *ultra-micro scale*, in contradistinction to the hitherto commonly used micro-analyses on e. g. phosphorus that deal with *the order of magnitude  $1\gamma$*  which will therefore in the following be referred to as the microgram scale or simply *the micro-scale*.

Among the most sensitive of the colour-reactions are the coeruleo-molybdate methods for the analysis of phosphorus and the pyridyl- and phenanthroline-reactions for the determination of iron. The limits of error for the Zeiss-Pulfrich photometer used

in conjunction with 1 cm. micro-cuvettes are given as  $3 \cdot 10^{-2}\gamma$  phosphorus (90) and  $2 \cdot 10^{-2}\gamma$  iron (65), which means that the tenfold amounts can be determined to within 10 %. For the determination of nitrogen there is a colour-reaction described by BERTHELOT that is about equally sensitive.

Before these reactions can be used for single large cells or a small number of little cells the sensitiveness must clearly be increased at least one hundred times. This increase in sensitiveness is a theoretical possibility, as is shown below. The required volume for a 1 cm. micro-cuvette used with a Zeiss-Pulfrich photometer is 0.2 ml., and if this volume contains  $0.3\gamma$  phosphorus, then the light-absorption in the above-mentioned colour-reaction will be about 40 % and it can be determined with an error of at the most 5--10 %. If one now diminishes the thickness of the layer to 1 mm., the light-absorption will be only 5 %, but this absorption can be measured with an error of only 0.02 % (i. e. 0.45 % error in the extinction, see e. g. 33). If the reaction is carried out in a volume of  $1\mu l^2$  it is already possible herewith to increase the sensitiveness of the method 200 times. With a photometric error of only 0.02 % in the absorption it would even be possible to diminish the thickness of the layer to 0.1 mm., and thus also the volume by a power of ten, and still get just as good measuring results on this micro-scale as with the Pulfrich photometer. This would mean increasing the sensitiveness by 2000 times, which would suffice for many cyto-chemical problems.

In order to be able to extend the measuring range of the photometric methods to cell-dimensions according to the above principles, it is clearly necessary to have firstly a sufficiently sensitive apparatus for micro-photometry, secondly a technique for the manipulation of the small sample-quantities, and thirdly a suitable method for the pre-treatment of the samples and for the actual execution of the colour-reaction, that must take place in the least possible volume. These essential conditions will be dealt with in the following chapters. And finally, a satisfactory histological technique is required for the taking of the samples. This is described in connection with applications on biological material in part II.

Two different procedures may be resorted to for the carrying out of the necessary pre-treatment of a sample to enable a colour-reaction in the least possible volume. In the one, the pre-treatment is

\* The symbol  $\mu l$  is employed to designate a millionth part of a liter (corresponding to the older designation cubic millimeter).

performed in a relatively large volume. The substance to be determined is then isolated, e. g. by precipitation, distillation or the like, after which the colour-reaction may be carried out in the volume desired. The advantage of such a technique is that the preliminary operations can be performed on a handier micro-scale. It is, however, necessary that the isolation should be achievable without too great a margin of error.

The other possibility is to work throughout with the smallest possible quantities, though this requires greater accuracy in the technique to keep the margin of error within the necessary limits.

As the isolation of phosphate would clearly entail great difficulties, the other alternative was chosen as the more suitable. In principle the analysis was thus to be carried out according to the following *general schema*: 1. Isolation of the sample on a fine needle of suitable material (e. g. quartz). 2. Pre-treatment on the needle (e. g. extraction, mineralization, etc.), if necessary with the sample in a pendant drop, that is afterwards allowed to dry, so that the sample now remains as ortho-phosphate in the dry state. 3. Colour-reaction in pendant drop. 4. Transference to a suitable medium in a micro-cuvette and photometry.

### CHAPTER 3.

#### **Apparatus for micro-photometry and its use.**

According to the foregoing discussion, a sufficient sensitiveness in the photometry is a *conditio sine qua non* for the exploitation of the volume-decrease and for the extension of the practical range of the colour-reactions to cyto-chemical problems. The greatest possible photometrical accuracy can be obtained with photo-electrical apparatuses where the photo-cell arrangement serves as zero-indicator, while the actual measuring takes the form of optical compensation — the so-called substitution-method. Although several writers have described sensitive photo-electrical apparatuses, such apparatuses are only in exceptional cases sufficiently sensitive to allow of light-absorption measurements with an error of less than 0.1 %. A discussion of the conditions required to achieve high accuracies is given by DECK (46), and the most important view-points are also included in a number of



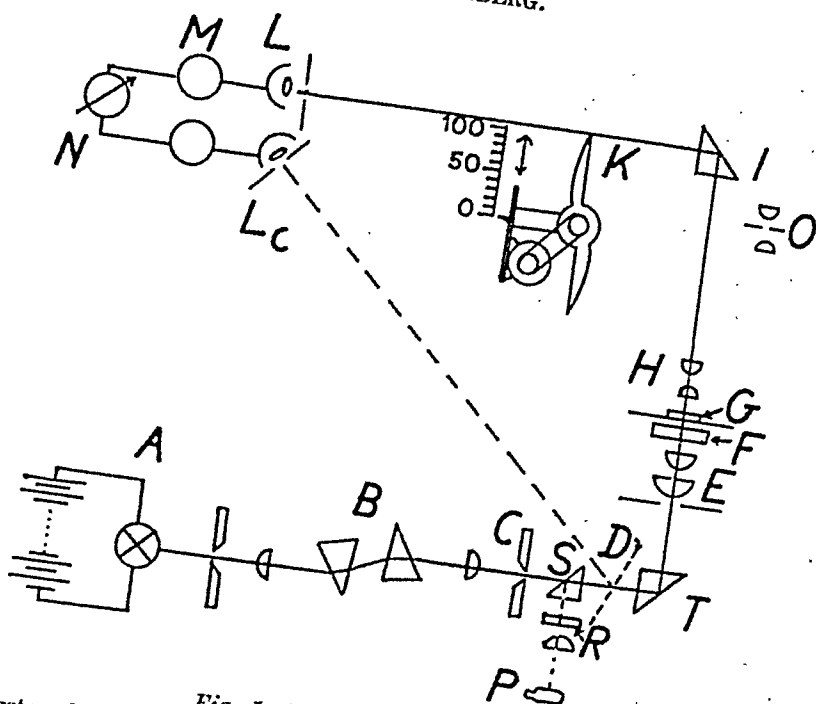


Fig. 1. The micro-photometer.  
 A, tungsten band-lamp. B, monochromator. C, second monochromator-slit.  
 D, glass plate (used with photo-cell  $L_c$  to compensate for changes in the mercury  
 lamp P). E, condenser. F, R, filters. G, object. H, objective. O, ocular inter-  
 changeable with prism I. K, rotating sector. L, photo-cell. M, amplifying circuit.  
 N, galvanometer. S, movable prism used with the mercury lamp P. T,  $90^\circ$  prism.

more recent surveys and original works (33, 71, 73, 74, 94), and it is therefore superfluous to go into them in detail here.

The apparatus for micro-photometry that I have used is schematically reproduced in figs. 1 and 3. The actual measuring is performed in this by optical compensation with a rotating sector; and a photo-cell amplifying circuit with galvanometer is employed as zero-indicator. This apparatus allows of a very great reproducibility of the measurements, and it probably represents a practical limit for sensitiveness of measurement, or at all events one that cannot be bettered without much extra work in construction and metrological technique.

*Description.* The chief parts of the apparatus consist of the source of light A with monochromator B, the microscope H with the object G, the light-subduing arrangement K and the amplifying circuit for the light-measurement L, M with the galvanometer N. The source of light normally consists of a 100 watt tungsten band-lamp that is fed from an accumulator battery with a large capacity (150 to 200 ampère-hours). By this means dis-

turbing variations in the strength of the source of light during a measurement are obviated. For special cases a high pressure mercury lamp P (Philips Philora HP 300) with spectral filter R is employed, a 90° prism S being inserted between the exit-slit of the monochromator and the semi-reflecting glass D.

The monochromator is of Winkel-Zeiss manufacture. The wave-length range  $2d$  for one slit-unit was determined with a number of mercury-lines in the usual way. The values obtained are given in table 1. If the slits are on  $i$  (entrance-slit) and  $u$  (exit-slit)

Table 1.

*Control of the purity of the light in the monochromator.*

Mercury line used.	Range of wave-length found with slit on 5.	$d$ for 1 slit unit.
6907 Å . . . . .	6640—7300 Å	66 Å
6234 » . . . . .	6100—6490 »	39 »
{5790 » . . . . .	5410—6150 »	37 »
{5769 » . . . . .		
5461 » . . . . .	5190—5730 »	27 »
4358 » . . . . .	4220—4480 »	13 »
4046 » . . . . .	3940—4140 »	9 »

units, the exit-slit will let through a wave-length range that is = set wave-length  $\pm (i + u) \cdot d$ . If one only reckons 75 % of the light-intensity the calculation is simplified to wave-length range = set wave-length  $\pm i \cdot d$ . The slits should be set equally. With both the slits on 1.5 units, which is the adjustment that applies where nothing to the contrary is stated, the wave-length range that was let through was controlled with a grating-spectroscope. The values obtained, together with those calculated as above, are given in fig. 2. As the monochromator when adjusted to 7200 Å showed green surplus light, a filter F, that completely absorbs all light more short-waved than 5700 Å, was inserted for the phosphate analyses.

The optical arrangements comprise an achromatic condenser E and an apochromatic objective H num. aperture 0.30 as well as total-reflecting prisms I, S, T. For adjusting the object an ocular O is inserted, that is removed during measurement.

The light-subduing arrangement. To begin with, a rotating sector cut out of sheet-aluminium was used. The sector with motor was

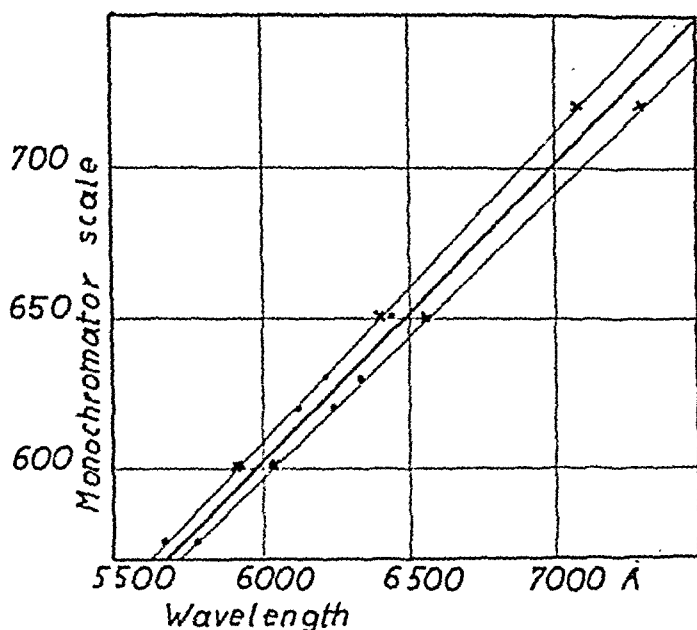


Fig. 2. The correlation between the monochromator-scale and the wave-lengths of the transmitted light. Both slits on 1.5 units (x calculated values — see text —, • values found with the grating-spectroscope).

shifted on a slide with rack and pinion-wheel, so that the necessary amount of light was screened off, and the reading could be made during the rotation of the sector on the scale attached thereto (figure 1, K). This method will serve if no great degree of accuracy is required. When measuring with two such sectors with 10 % and 20 % absorption respectively for 100 mm. displacement the metrological variations are found to be in the vicinity of 0.2 % (total absorptions from 2 to 14 %). In order to get the best results with this arrangement the ray of light must at the passage of the sector be as narrow as possible. For physical reasons it was not possible, in the measurements mentioned, to place the sector in such a way that the ray of light took up less than 3 mm. on the sector. With a better placing of the sector one may thus count upon greater accuracy even with this simple arrangement.

These difficulties are entirely eliminated by the use of a rotating sector consisting in principle of two discs rotatable in opposite directions and with two 90° segments removed. With such a sector the intensity of the passing light can be varied from 0—50 %. The type of sector now used in the apparatus is constructed after the pattern of the excellent sector from the Askania-Werke in Berlin. Adjustment and reading may be made while the sector is



As zero-instrument a Zernike c galvanometer (Kipp & Zoonen) is used. With this arrangement the stability-level is  $3 \cdot 10^{-16}$  ampères with a tension-sensitiveness of 10000 scale-divisions per volt. By means of an Ayrton-shunt, V, the sensitiveness can where required be reduced to 0.1 and 0.01 of that given above. It proved difficult, at first, to avoid external interferences, for the building in of the amplifier separately and the batteries separately in a cupboard with earthed sheet-metal walls proved inadequate. The whole amplifying unit was then built into a Faraday's cage with only one earthing between the two screens. This resulted in very satisfactory freedom from interference. For the stability it also proved necessary to diminish the number of unsoldered contact-spots, and in those cases where unsoldered contacts were necessary they were designed as frictional contacts with large contact-surfaces and several contact-springs. After the introduction of these arrangements the amplifier functioned flawlessly in daily routine.

*Photo-cells.* Photo-cells designed for high isolation and filled with inert gas are employed. For the wave-length region 4500—5500 Å potassium-cells are used, and for the long-wave part of the visible spectrum potassium-caesium-cells are employed.

*The performance of the measurement.* Half an hour before commencing the measuring the filament current is switched on to the amplifier, and, if the accumulators are newly charged, also to the tungsten band-lamp. Before the measurement the object is so adjusted that the image of the exit-slit of the monochromator appears in the middle of the object. The size of this image is  $0.12 \times 0.12$  mm. The slit-image is projected right over the photo-cell opening ("the measuring-cell") by the objective and the prism I, which is movable about an axis in the horizontal plane and an axis in the vertical plane. The sector is started, and the shutter in front of the measuring-cell is opened. In order to compensate the photo-current a special compensation-potential is added to the grid of the other photo-cell ("the compensation-cell") with the help of the potentiometer U (fig. 3) until a definite galvanometer reading is obtained, suitably the same reading as that which the galvanometer showed before the measuring-cell was illuminated. After 1—2 minutes the galvanometer reading is generally constant to within 0.5 mm. The object is now removed with the help of a measuring stage, so that the light is instead allowed to pass through the pure solvent (or suitable blank).

The light-absorption of the sample is now *substituted* by an equal light-absorption from the sector, whereupon the galvanometer-reading remains the same as the reading that has previously been determined. In order to eliminate casual errors the measurement is performed several times with the same object. If the absorption of the sample is on an average  $x\%$ , then the extinction

$E = \log \frac{100}{100 - x}$ . The reproduceability in connection with measurements of the same object is of the same order of magnitude as the reading-accuracy of the sector, i. e.  $0.025\%$  in the absorption. The photometric accuracy is thus sufficient for measurements of light-absorption down to  $1\%$  with an error of only about  $2\%$  in the extinction.

The above description applies for measurements in connection with which the tungsten band-lamp with large accumulators is used, in which case the variations in the light-intensity from the lamp are so small that they do not affect the result if the measuring is done rapidly. In case of varying light-intensity (mercury-lamp) the apparatus is used as a two-cell photometer, a change that does not entail any re-wiring. The photo-current in the measuring-cell is here compensated with an equally great photo-current from the compensation-cell Lc by illuminating the latter with a part of the light (from the semi-reflecting glass D, fig. 1). Variations in the light-intensity of the lamp hereby affect both the photo-cells alike, and will therefore not disturb the measurements. Thus, when measuring with the apparatus as a two-cell photometer the compensation-current from the potentiometer U (fig. 3) is replaced by an equally great photo-current from the compensation photo-cell. The measurements are otherwise carried out in the same way as described above.

## CHAPTER 4.

### The sample-volume and the application of the sample for photometry.

In chapter 2 it was unconditionally assumed that one could diminish the reaction-volume as far as the optical measurement allowed. Certainly one can, as BENEDETTI-PICHLER (11) has shown, handle volumes of the order of magnitude  $10^{-8} \mu\text{l}$ , but the requisite technique is in this connection of interest only as a curiosity. CASPERSSON (19) has treated in some detail the prime conditions for photometry in small drops, and has even succeeded in carrying

out such measurements with volumes of about  $10^{-6}$   $\mu\text{l}$  (33). But even if it is thus possible both to pipette and photometrically measure such small sample-volumes, a technique based on this would probably be subject to very great errors owing to difficulties of manipulation, and it would certainly not be suitable for series of determinations. It thus seemed advisable from the outset to try to use such large volumes as could be handled more or less quickly and surely, and the order of magnitude 1 to 0.1  $\mu\text{l}$  was therefore chosen. In choosing the method of pipetting, a type of pipette was first tried from which the liquid was driven out by electrical warming. Experiments were also made with a type of pipette based upon mechanical pressure with a piston. In neither case was the accuracy obtained greater than that which can be attained with hand-pipettes of Carlsberg type (see 89), that with only 1  $\mu\text{l}$  can show a pipetting error as slight as 1 %. To illustrate the pipetting accuracy with hand-pipettes in sizes from 0.1 to 2  $\mu\text{l}$  some results have been assembled in table 2. With volumes as low as 0.1  $\mu\text{l}$  the error<sup>3</sup> is, however, as emerges from the table, so great as already to constitute a practical limit, unless it is possible in some other way to measure the volume with greater accuracy.

Theoretically it would be possible to solve this problem by adding to the sample a definite amount of a substance with strong light-absorp-

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<sup>3</sup> For the statistical treatment of the analytical results the following methods are employed:

1. The dispersion  $\sigma$  is calculated according to the formula  $\sigma = \pm \sqrt{\frac{\sum a^2}{n-1}}$ , where  $a$  = the deviation from the mean value and  $n$  = the number of observations.

2. The variation-coefficient  $\nu$  is calculated according to the formula  $\nu = \frac{100 \cdot \sigma}{M}$ .

3. The mean error  $\epsilon(M)$  of the arithmetical medium  $M$  is calculated according to  $\epsilon(M) = \pm \frac{\sigma}{\sqrt{n}}$ .

4. The mean error  $\epsilon(D)$  for a difference  $D$  between two mean values  $M_1$  and  $M_2$  is calculated according to the formula  $\epsilon(D) = \epsilon(M_1 - M_2) = \pm \sqrt{\epsilon(M_1)^2 + \epsilon(M_2)^2}$ .

5. A difference  $D$  between two compared mean values  $M_1$  and  $M_2$  is regarded as

"none" if  $\frac{D}{\epsilon(D)} < 2$

"probable" if  $\frac{D}{\epsilon(D)} \geq 2$  but  $< 3$

"certain" if  $\frac{D}{\epsilon(D)} > 3$ .

<sup>4</sup> For the concentration units the following abbreviations are used:  
 Molar concentration = mol. Millimolar concentration = mmol.  
 Normality = n. Millinormality = mn or meq./liter.

Table 2.

Calibration of micro-pipettes. With the pipette a  $\text{NaIO}_3$  solution was put into excess  $\text{H}_2\text{SO}_4 + \text{KI} + \text{starch}$  and the  $\text{I}_2$  titrated with  $\text{Na}_2\text{S}_2\text{O}_3$ .

Pipette no.	1	2	3	4
$\mu\text{l Na}_2\text{S}_2\text{O}_3$ . . . . .	4.52 4.89 4.84 5.81 5.25 4.66 4.79 4.51 4.77 4.79 5.17 4.66 4.44 5.55 4.69	15.60 16.05 15.66 15.14 15.62 15.25 15.21 15.39 15.58 15.17 15.52 15.41 15.58 15.36 15.53 15.63 15.36 15.32 15.18	35.79 36.24 36.14 35.99 37.05 36.15 36.22 36.17 36.49 35.83 36.12 36.19	14.82 14.28 15.07 15.01 14.86 14.76 14.84 14.63 14.67 14.32 14.90 14.72
Ditto mean . . . . .	4.89	15.45	36.20	14.74
$\sigma^*$ . . . . .	0.39	0.23	0.33	0.30
$\text{Na}_2\text{S}_2\text{O}_3$ milli-normality† .	25.9	25.9	25.6	25.7
$\text{NaIO}_3$ ditto . . . . .	925	925	925	185
Pipette volume $\mu\text{l}$ . . .	0.137	0.433	1.00	2.05
$\nu^*$ . . . . .	8 %	1.5 %	0.9 %	2.0 %

\* See footnote 3) † See footnote 4) p. 16.

tion that does not collide with that of the sample nor affect the sample's colour-reaction. Such experiments have been made with neodymium nitrate and the coeruleo-molybdate reaction; but they have not led to any improvement on the present stage of the method.

Besides the pipetting difficulties there is, however, when working with these small volumes, another factor of great importance to be considered, namely, the evaporation. This was tested by weighing drops of water that were hanging on a glass needle of the same type as was afterwards used for the micro-phosphate determinations. As the experiments in table 3 show, the volume-variations due to the evaporation are considerably greater than



Table 3.

*Loss in weight per minute from water droplets of different sizes.*

Volume $\mu$ l:	0.114	0.442	1.00	2.05
Loss in weight per minute mg. . . . .	0.032	0.048	0.060	0.075
	0.034	0.056	0.074	0.080
	0.030	0.056	0.066	0.075
	0.036	0.046	0.066	0.080
	0.030	0.050	0.060	0.080
	0.030	0.046	0.064	0.090
	0.036	0.056	0.052	0.090
	0.032	0.060	0.058	0.090
	0.028	0.056	0.056	0.085
	0.024	0.050	0.059	0.080
		0.054	0.065	0.085
		0.052	0.070	0.090
		0.042	0.066	
		0.045		
Ditto mean . . . . .	0.031	0.051	0.063	0.085
Ditto % of total weight .	27	11.5	6.3	4.15

those due to the pipetting. If one reckons that the sample-drop is exposed during the performance of the colour-reaction to the influence of the air for 30 to 45 seconds the pipetting and evaporation errors even for 1  $\mu$ l could amount to about 5 %, and rise rapidly for smaller volumes. The extension of the practical range of the method downwards that the use of smaller volumes would represent would thus be illusory. The practical volume-limit for the method is therefore set at about 0.5—1  $\mu$ l.

For the photometrical application of the sample-drop CASPERSON (33) used cyclohexane as an indifferent medium, while BENEDETTI-PICHLER (loc. cit.) worked with a pendant drop on a glass covered with nitro-cellulose and with paraffin oil as indifferent medium. Besides these media, also tricresyl-phosphate, triethyl-citrate, diethyl-phthalate and benzyl-benzoate were tried; but as these did not offer any advantage over paraffin oil only liquid paraffin was used thereafter. My own experiments also showed the necessity of covering the glasses with a hydrophobic layer to prevent the small drops from flowing out. A very good hydrophobic layer could be obtained with a film of highly nitrated cellulose to which certain plasticisers were added.

Nitro-cellulose with a nitrogen-content of 12.2 % ("CN") and 13.45 % ("CN<sub>2</sub>") was tried in different solvents and with about ten different plasticisers. In order to get an optically satisfactory layer it proved suitable to employ solvents with rates of evaporation of 11—14 when that of ethyl-ether is = 1 (DURRANS loc. cit., p. 48), e. g. n-butyl-acetate, n-butyl-propionate or amyl-acetate. Suitable plasticisers were acetophenone, tricresyl-phosphate, benzyl-benzoate, dialkyl-phthalate (ethyl-, butyl- and amyl-), especially in combination with butyl-stearate, triethyl-citrate or dibutyl-tartrate. These were in the main used in the proportions given in DURRANS' monograph.

On such a hydrophobic layer the drops assumed an almost semi-spherical form, which could, moreover, be shown by measuring the diameter at the base. A couple of examples are given in table 4.

Table 4.

*Measurement of the diameter at the bases of water droplets of known volumes on different hydrophobic layers. Volume 0.442  $\mu$ l, diameter calculated for hemispherical shape 1.189 mm. The cellulose nitrate for the hydrophobic layers was plasticized with diethyl-phthalate and butyl-stearate. Butyl-propionate (BuPr) or amyl-acetate (AmAc) were used as solvents.*

Hydrophobic layer	CN, BuPr	CN, AmAc	CN <sub>2</sub> , BuPr
Scale-divisions in the ocular micrometer . . . . .	320	328	342
	326	344	314
	331	323	318
	327	327	313
	337	330	314
	334		325
	340		319
	333		
	338		
	329		
	324		
Ditto mean value . . . .	331	334	321
Diameter found mm. . .	1.32	1.33	1.28

Although the drops thus assumed a favourable form for photometry, the measurements proved to be less accurate than measurements in cuvettes, a circumstance that is probably chiefly due to the difficulty of defining the layer-thickness for the drops. For the measurements in cuvettes 1 mm. spectrograph cuvettes were

employed, so that the thickness of the layer was well-defined. Since, however, 1 mm. is quite a considerable layer-thickness and requires a volume of several  $\mu\text{l}$  even if end-plates with hydrophobic layer are used, the following technique was tried, and afterwards employed as a routine method: on an object-glass with hydrophobic layer are placed two narrow strips of a polished covering-glass (hemocytometer-glass) of a thickness of e. g. 0.35 mm. Between the pieces of covering-glass are then pipetted sample and blank-drops, after which paraffin-oil is dripped on and the whole is covered with covering-glass. With this technique, even with sample-volumes of only 0.15  $\mu\text{l}$ , I obtained good adhesion to the glasses.

For the checking of the layer-thickness one may resort to different procedures. For routine use the following technique proved suitable. Transversely across the object-glass one draws with Indian ink two thin lines, with a space of 1 cm. between. Similar lines are then drawn on the covering-glass, with an interval of three to five mm. between. The covering-glass is laid on the object-glass in such a way that the lines on the two glasses intersect. One thus has four points of intersection around the sample-drop, where the distance from the underside of the covering-glass to the upper side of the object-glass can be measured by adjusting the microscope on the two inked lines with the aid of the micrometer-screw. The number of  $\mu$  according to the micrometer-screw multiplied by the refraction-index of the medium gives the layer-thickness. The accuracy of this determination was tested by measuring a glass with inked lines on both sides and comparing the calculated thickness with that measured with the micrometer-screw. In other experiments spectrograph cuvettes of 0.33 mm. thickness were employed, with paraffin and inked lines on the end-plates. The results of this test appear in table 5.

In order to check the suitability of these simple micro-cuvettes, the pre-treatment of the glasses and the layer-measurements, comparative experiments with photometry were carried out in a Zeiss-Pulfrich photometer and in the new micro-photometer. For these experiments the coeruleo-molybdate reaction according to FISKE-SUBBAROW (55) was used, with the reagent-proportions given by TEORELL (116). The readings were always carried out between 30 and 90 minutes after the mixing, a period during which no significant changes in the extinction could be observed. The results may be seen in table 6, which shows that the photo-

Table 5.

*The accuracy of the method for measuring the thickness. Measurement in micro-cuvette with paraffin ( $n = 1.433$ ) and on glass-plates ( $n = 1.51$ ) with inked lines on both sides and controlled with covering-glass micrometer. Measuring objective achromatic 40 num. ap. 0.65. Micrometer-screw of microscope divided in 0.002 mm.*

Object	Cuvette	Glass	Glass	Glass
Thickness of layer . . . .	0.328 mm	0.270	0.375	0.385
Scale-divisions on micrometer-screw . . . . .	116.5	92	126	127
	118.5	89	127	129
	114	89	126	129
	117	91	126	130
	113.5	91	125	130
	116.7	92	127	128
	115.5	91	122	127
	116.3	91	125	129
	116.5	88	125	126
	116.5	88	126	129
	116	91	127	130
	116.7	88	127	130
	117.5	88	127	132
	116.5	88	124	131
	115.7	90	125	127
	117.3	92	123	128
	117	93	123	130
	116.7	89	124	131
	116.3	88	127	131
	115.7	93	125	131
	116.7	90	125	132
	117	89	125	132
	117.5		125	132
	116.7		125	131
	117		124	131
	116.5		124	131
	117.5			
Mean value . . . . .	116.4	90.0	125.0	129.8
$\sigma$ . . . . .	1.04	1.7	1.4	1.7
Empirical thickness of layer	0.333 mm	0.272	0.377	0.392

metrical accuracy on the ultra-micro scale is about as great as with the Zeiss-Pulfrich photometer, although the absorption in the former case is less than  $\frac{1}{15}$  of that found in the latter.

The experiments show, moreover, the excellent reproduceability

Table 6.

Comparison between photometric micro-determination of phosphorus (the Pulfrich photometer) and ultra-micro determination according to the technique described. Coeruleo-molybdate method: Fiske-Subbarow (116). Samples of 0.13  $\mu$ l were taken from mixtures with the final concentrations given, placed in micro-cuvette and measured against blank samples at 720  $m\mu$ , i. e. 708—730  $m\mu$  (fig. 2) in the micro-photometer. The same mixtures were also measured in the Pulfrich photometer with filter S. 72. The table contains the calculated values of the specific extinction-coefficient.

$\gamma$ P/ml	1.86	2.48	3.10	3.72	4.34	4.96	5.58	9.3
Ultra-micro scale.	141	154	128	143	139	138	137	135
	129	145	129	137	133	151	132	139
	134	146	132	132	123	132	124	135
	133	145	134	136	135	134	123	139
	145	145	120	136	126	131	132	135
	149	127	135	132	132	139	129	140
	144	146	134	137	141	135	134	137
	151	140	127	133	144	153	132	135
	147	139		138	148	151	136	138
	138	141		140	139	134	133	136
	146	139		136		147	136	140
	142	139		141		146	141	
Mean value . . .	142	141	130	137	136	142	132	137
$\sigma$ . . . . .	6.6	6.6	3	3.5	7.8	8.3	5.2	2.3
Micro-scale . . .	134			138			137	
	142			141			136	
	136			138			138	
	142			141			138	
	136			144			143	
	146			148			145	
	140			140			140	
	145			145			141	
	135			136			137	
	138			140			137	
	135			136			138	
	133			133			138	
Mean value . . .	138			140			139	
$\sigma$ . . . . .	4.5			4.2			2.7	

of this phosphorus method; for although they were performed at different times and the reduction reagent ("ANS") was renewed a couple of times in the series, the value of the extinction-coeffi-

cient<sup>2</sup> is in this case constant within the limits of error. As the agreement in respect of the specific extinction-coefficient between the results found with the Zeiss spectro-photometer and those found with the ultra-micro method is so convincing, it is clearly not necessary to control the specific extinction-coefficient with micro-photometry for each new lot of reagents; this control can be made with the Pulfrich photometer.

Although FISKE-SUBBAROW'S coeruleo-molybdate method is undoubtedly very good under favourable conditions, it has not become the dominant method for the photometric micro-determination of phosphorus; a number of other coeruleo-molybdate methods have been extensively employed. This is partly due to the fact that many authors have obtained unsatisfactory results with FISKE-SUBBAROW'S method, and have therefore gone back to some older method or worked out a new one. A critical investigation of the most important coeruleo-molybdate methods is therefore called for. This will be given in chapter 5.

## CHAPTER 5.

### The colour-reaction.

The colour-reaction that one desires to use for the micro-photometric determination of a substance should fulfil certain requirements. First of all, the reaction should be specific or at least strongly selective for the substance in question. Further, the colour should be proportional to the amount of sample used and should follow the general absorption law (Lambert-Beer's law). The colour development should proceed rapidly to a constant level. It is also desirable, as regards the micro-photometric procedure, that the reaction should be able to be carried out with the fewest possible manipulations on account of the risk of evaporation (see table 3, p. 18), and that the specific extinction-coefficient should be high, whereby smaller quantities can be determined. And finally, in the ultra-micro procedure the milieu must not be allowed to influence the course of the reaction, or in others words,

<sup>2</sup> According to the absorption law we have  $E = \log \frac{I_0}{I} = k \cdot c \cdot d$ , where  $E$  is the extinction,  $I_0$  is the intensity of the incident light, and  $I$  is the intensity of the light after its passage through the sample.  $k$  is the specific extinction-coefficient,  $d$  is the thickness of the layer in cm. and  $c$  is the concentration. In the following,  $k$  is always calculated for the concentration-unit 1 mg. per ml.

the reaction must proceed in the same way as on the microgram scale.

The coeruleo-molybdate reaction on ortho-phosphate can be carried out in several different ways as regards the reducing agent etc. In all cases, however, it holds good that the reaction should be not specific but in a high degree selective. It is otherwise given only by arsenate and silicate. Other phosphoric acids do not give a colour unless the milieu is strongly acid and the temperature high, in which case they may change into ortho-phosphoric acid and react (14). By means of special modifications of the methods, however, the effect of arsenate and silicate may be avoided, so that the determination is specific.

As regards the validity of the absorption law, it is theoretically possible for deviations to occur, either through the reaction taking a different course with different quantities of sample or through the presence of other substances or through the reduction of excess molybdate (see below), or else because the specific extinction-coefficients for the wave-lengths used in the measuring are very different (see KORTÜM, 71, 73, 74). The form of the absorption-curve in the coeruleo-molybdate reactions is, however, so flat that no deviations from the absorption law are obtained hereby, despite the relatively large wave-length range that is used in the Pulfrich photometer (filter S. 72), and still less is this the case in the ultra-micro procedure. Concerning substances that interfere with the different reactions, there are a number of data in the literature (3, 12, 45, 109, 120, 131 and others). In the specific case here in question, however, the risks of disturbing effects by foreign elements are small. Organic substances are destroyed by incineration, so the substances that might interfere are inorganic salts. These occur, however, in such small quantities, that they do not, in fact, affect the reactions.

The absorption-curve for the blue phospho-molybdic complex was measured with a photo-electric spectral photometer. The spectral bands employed had a breadth of 14  $m\mu$  at 560  $m\mu$  and up to 28  $m\mu$  at 760  $m\mu$ . The mean values of the specific extinction-coefficients,  $k$ , from some such measuring series are given in table 7.

Table 7.

*The absorption-curve for coeruleo-molybdate between 560 and 760  $m\mu$ .*

Wave-length $m\mu$ . . . . .	560	580	600	620	640	660	680	700	720
Specific extinction-coefficient .	82	87	100	104	118	123	130	135	136
Wave-length . . . . .	740	760							
Spec. ext.-coeffic. . . . .	135	133							

A factor of primary importance in the choice of method is the sensitiveness, that may be expressed with the specific extinction-coefficient. This shows considerable differences in the different coeruleo-molybdate reactions. A survey of the different methods shows that these can be divided up into three groups, to wit, those that can be carried out with organic reducing agents, those that employ previously reduced molybdic acid, and those that are carried out with inorganic reducing agents. The list given below shows specific extinction-coefficients for some of the most commonly used methods.

Author	Spec. extinction-coefficient k	Reduction
FISKE-SUBBAROW (55) . . . . .	approx. 150	Amino-naphthol-sulphonic acid
BELL-DOISY (10) . . . . .	» 140—225	Hydroquinone
ZINZADZE (131) . . . . .	» 400—500	Of the reagent with molybdenum
DENIGÈS 1927 (49) . . . . .	» 400—500	Of the reagent with copper
DENIGÈS 1920 (47) . . . . .	» 700	Stannous chloride
BERENBLUM-CHAIN, STOLL (13, 114)	» 700	» »

Of these methods it will thus be seen that FISKE-SUBBAROW's is the least sensitive, although it is actually a very sensitive photometric method. According to URBACH (123, 124), BELL-DOISY's method has a rather higher specific extinction-coefficient,<sup>6</sup> but this added sensitiveness is counteracted by certain disadvantages. In this method the phospho-molybdic acid is formed first in acid milieu, as is always the case, after which, however, the reaction is concluded in alkaline milieu. This entails in the first place from the micro point of view unfavourable manipulations, and in the second place a risk that silicic acid may go into solution from the glass or quartz needle on which the sample is pendant. This method has therefore not been included in the tests.

A method that is intended entirely to obviate the possibility of reduction of any molybdate surplus has been described by ZINZADZE (131). The reagent used in this method is a pure coeruleo-molybdate complex that does not contain any reducing agent and that is strongly blue-coloured. On dilution the complex is broken down or changed in some other way, so that the colour disappears.

<sup>6</sup> According to TSCHOPP and TSCHOPP, the colour on reduction with hydroquinone is fainter than with 1, 2, 4-amino-naphthol-sulphonic acid, and ZAMBOTTI gives the specific extinction-coefficient as 142.



If phosphorus is present, however, the blue colour reappears, though not without the addition of a certain warmth. As the method is very sensitive, gives a very stable colour and is simple to perform, it seemed promising for adaptation to the ultra-micro scale and was therefore submitted to a closer investigation.

Another very sensitive method, that like ZINZADZE's makes use of a previously partially reduced reagent, was described as early as 1927 by DENIGÈS (49, 50). Also this method has been investigated in respect of its suitability for ultra-micro application.

Finally, the most sensitive of all coeruleo-molybdate methods are those that are performed with stannous chloride as the reducing agent. Of these, the classical method according to DENIGÈS (47, 48) has been thoroughly tested in various forms. Two more modern methods should also be mentioned here. They have been described by BERENBLUM and CHAIN (13) and by STOLL (114) respectively. In both of these methods certain sources of error (12) are avoided by the isolation of the phospho-molybdic acid before reduction by extraction with isobutyl-alcohol and ethyl-acetate respectively. These methods are not, however, suitable for the ultra-micro scale, as the necessary manipulations — extraction, washing, reduction with separations between the different phases — even if it should be possible to perform them quantitatively on the necessary micro-scale, would in any case introduce so many new sources of error that the advantages of the procedure would prove illusory — apart from the great increase in the analytic work.

Other conditions for the suitability of the reactions will be discussed in connection with the detailed investigation of each method below.

\*            \*            \*

*Fiske-Subbarow's method.* — The method of FISKE and SUBBAROW was described in 1925, and it has since then been extensively used in biochemistry and in other spheres in a number of different modifications (see TSCHOPP and TSCHOPP, 120, MANLY, 92). As I have over a number of years had a favourable experience of the method in TEORELL's modification (116), I have employed it in the working out of the ultra-micro technique. The colour in the reaction is strictly proportional to the amount of phosphorus within a big concentration-range (see table 6, p. 22). The colour, that is little sensitive to slight temperature variations ( $\pm 2-3^\circ$ ),

leads at room-temperature within 20 minutes to a colour-level that subsequently remains unchanged for an hour and a half. Normally, the reaction is performed as follows: to the sample is first added molybdate and sulphuric acid, so that phospho-molybdic acid is formed, after which the reducing agent is added. This would be a certain disadvantage for the ultra-micro scale, as has been discussed in detail above. As there is reason to assume that phospho-molybdic acid need not be formed before the reduction, but that the colour development depends upon a complex formation between the 6-valent and the 5-valent molybdenum-oxide and the phosphate, in which connection the phosphate acts as a catalyst (FEIGL, 53, see also 12, 120 and others), it should be possible to mix the molybdic acid and the reducing agent before the addition of phosphate. It proved, also, just as effective to use only *one* reagent (in the following pages referred to as "compound reagent")<sup>7</sup> that immediately before use was compounded of sulphuric acid, molybdate and reducing reagent in the same proportions as in the normal procedure. This is shown by the experiments in table 8.

FISKE-SUBBAROW's reaction can, however, give bad values if the reagents are of poor quality. So, for example, the 1, 2, 4-amino-naphthol-sulphonic acid ("ANS") used in the present case (British Drug Houses) gave excellent values, while on the other hand a preparation from another manufacturer gave neither constant values for the specific extinction-coefficient nor in fact any reproduceable results at all. The only practical course to adopt in this connection is to try out different preparations until one finds one that is suitable. The preparation of the reagent has always been carried out in the following way. 12 g of sodium metabisulphite is dissolved in 80 ml of water, and 0.2 g of amino-naphthol-sulphonic acid is stirred in, after which 2 ml of 20 % crystallized sodium sulphite is added. This mixture is left to stand for some hours — generally over-night — after which undissolved amino-naphthol-sulphonic acid is filtered off. The reagent is kept in a dark bottle. According to the original description, sodium sulphite is to be added until all the amino-naphthol-sulphonic acid has gone into solution. A reagent prepared in this way did not keep so well and offered no advantages as compared with the one I employed.

<sup>7</sup> In certain modifications of FISKE-SUBBAROW's method the reducing reagent must be added *before* the molybdate, which makes two lots of reagent inevitable (3, 107).

Table 8.

Comparison between Fiske-Subbarow's method, normal procedure (116), and the same method with "compound reagent", i. e. the dry sample dissolved in a reagent of 1.5 ml 12.5 *n*  $H_2SO_4$  + 2.0 ml 5.5 %  $Am_2MoO_4$  + 1 ml amino-naphthol-sulphonic acid reagent + water to 25 ml. Values: spec. extinction-coeff., *k*.

P in $\gamma$ in 25 ml	k (compound reagent)	k (normal procedure)
31 . . . . .	153	139
	148	144
	146	142
	148	146
	145	158
	152	142
	136	141
	149	138
	141	
	149	
46.5 . . . . .	152	
	137	
62 . . . . .	147	158
	144	146
	145	140
	147	145
	147	144
	142	143
	143	
	138	
93 . . . . .	138	
	148	
	143	
	143	
	142	
Mean value . . .	146	
	145	145
	145	
	145	
	145	
$\sigma$ . . . . .	4.55	6.1
$\sigma'$ . . . . .	3.1 %	4.2 %

The purity, too, of the ammonium molybdate is of decisive importance. With a preparation designated "purissimum" it was not possible to obtain proportionality between extinction and phosphorus-concentration.

Owing to the difficulties under present conditions of obtaining analytically pure molybdenum preparations, it is advisable to regenerate

Table 9.

*Ultra-micro. determination of phosphorus as  $KH_2PO_4$ . The values give the specific extinction-coefficient  $k$ . Sample-volume 1  $\mu$ l.*

P in $10^{-3} \gamma$	1.75	3.50
$k$ . . . . .	137	153
	138	133
	166	151
	139	153
	164	150
	152	143
	154	155
	139	165
	149	161
	143	162
	158	159
	154	157
	162	139
		151
		156
Mean value . .	150	152
$\sigma$ . . . . .	10.4	8.8
$\nu$ . . . . .	7 %	5.8 %

the molybdate from the sample-solutions after the phosphorus-determinations. For this purpose the following method has proved suitable:

The blue solution, that contains a surplus of molybdic acid and coeruleo-molybdate in solution acidified with sulphuric acid, is evaporated to a suitable volume and decolourized with potassium permanganate. The sulphuric acid is then neutralized with ammonium hydroxide and the solution is filtered. The filtrate is saturated with sulphuretted hydrogen, after which all the molybdenum is precipitated as trisulphide  $MoS_3$  by acidification with hydrochloric acid. The molybdenum-trisulphide is taken on a filter and washed with distilled water and then dissolved in 10 % sodium sulphide. The reddish brown solution is filtered, and the molybdenum-trisulphide precipitated by addition of excess hydrochloric acid. The thus purified sulphide, that is obtained as 100 % yield, is placed on a filter, washed with distilled water and left to dry. The molybdenum trisulphide is now calcined in a slow oxygen current at 500–700°. The molybdenum-trioxide is dissolved in concentrated ammonium hydroxide and the solution evaporated in vacuum over solid sodium hydroxide. The crystallized ammonium-molybdate thus obtained (yield 65 % or more) was always of a satisfactory purity.

The molybdenum-content was determined cerimetrically according to

Table 10.

*Ultra-micro determination of phosphorus. The significance of the volume. The values give the specific extinction-coefficient  $k$ . The phosphorus concentration in all experiments =  $3.10 \gamma P/ml$ .*

Volume on photometry $\mu l$	0.46	0.46	1.0	2.05
$k$ . . . . .	136	175	137	129
	158	148	146	135
	179	153	136	143
	129	169	142	150
	118	183	152	142
	125	141	156	137
	144	170	144	149
	167	137	147	157
	100		129	150
	151		157	150
	133			142
				138
Mean value . . . . .	144	160	144	144
$\sigma$ . . . . .	23	17	9	8
$\nu$ . . . . .	16 %	10.6 %	6 %	5.6 %

FURMAN and MURRAY (56). The molybdenum-content was found to be approximately 49 %. Nitrogen was determined according to KJELDAHL and amounted to about 14.5 %. This corresponds to the compound  $Am_2MoO_4$  with 48.95 % molybdenum and 14.29 % nitrogen. The reagent-solution should therefore be 5.5 % in strength, i. e. 0.28 mol in respect of molybdenum, for 5 % ammonium paramolybdate is 0.28—0.30 mol in this respect.

Impure ammonium molybdate preparations may be purified in the same way, though possibly only *one* precipitation as  $MoS_3$  is required for this.

In order to test FISKE-SUBBAROW's method on the ultra-micro scale, experiments with the following arrangement were carried out. On needles of Jena Geräte glass known quantities of standard solutions of primary potassium phosphate were pipetted. When the solution had dried the phosphate was dissolved in a drop of compound reagent, and the sample-drop was put in a micro-cuvette (see chapter 4). Between 60 and 90 minutes after the commencement of the reaction micro-photometry was performed at 720  $m\mu$ . As table 9 shows, the sum of the errors arising in connection with pipettings, evaporation, measuring of thickness, colour-reaction and photometry (the mean error in a single de-

termination) is of the order of magnitude 10 % for amounts of phosphorus of  $1.75$  and  $3.50 \cdot 10^{-3} \gamma$ .

In order to control the importance of the evaporation, a series of similar experiments was carried out with solution in three different volumes. As table 10 shows, the greater evaporation-effect for the least volume as compared with the larger volume is evident from the greater mean error and the higher value of the extinction-coefficient. The latter is the case, however, only in the one series, so that the evaporation-effect is evidently not always necessarily so prominent as table 3 (page 18) might lead one to suppose.

*Summarizing*, one may say that FISKE-SUBBAROW's method is well adapted for phosphorus-determinations also on the ultra-micro scale.

A method that in respect of sensitiveness is comparable with FISKE-SUBBAROW's was worked out during an earlier period of these investigations on the basis of the reaction described by VAN DER MEULEN for qualitative use (93). The advantages of the method consist in the greater stability of the colour (table 11) and the low acidity of the reaction-milieu. The reagent is mixed immediately before use, the constituents being 4 ml of 1 mol  $\text{Na}_2\text{MoO}_4$  + 156 ml of distilled water + 10 ml of 1 n  $\text{AmF}$  + 10 ml of 0.1 n  $\text{FeSO}_4$  in 0.63 n  $\text{H}_2\text{SO}_4$ . One takes 4 volumes of this reagent to 1 volume of sample, so that the definitive concentration of sulphuric acid amounts to 0.035 n, or less than one tenth of the concentration according to FISKE-SUBBAROW's method.

Table 11.

*The coeruleo-molybdate reaction according to van der Meulen's principle.*

Reaction period hours	1	2	3	5	24
$\gamma$ P/ml	Specific extinction-coefficient				
1.24 . . . . .	149	145	149	146	150
1.86 . . . . .	144	143	158	158	151
2.48 . . . . .	146	148	150	157	159
3.72 . . . . .	145	147	149	148	150
4.96 . . . . .	147	147	156	149	150
6.2 . . . . .	132	145	152	149	148

An interesting modification of FISKE-SUBBAROW's method was described by ZINZADZE in 1935 (132). In this method the colour-development takes place at  $100^\circ$  for 30 minutes. The sensitiveness is said to be just as great as in DENIGÈS' stannous chloride method. A control-test showed, however, that the specific extinction-coefficient was

only something over half of the value according to the stannous chloride method. It varied considerably, moreover, both within the same series and from day to day. A further testing of this method for use on the ultra-micro scale was therefore considered uncalled for.

\*       \*       \*

*Zinzadze's method.* — The method of ZINZADZE was described in 1930, and it has since found application especially in agricultural chemistry. The reagent consists of a strongly  $\text{H}_2\text{SO}_4$ -acidified solution of molybdic acid,  $\text{MoO}_3$ , and the next lower oxide  $\text{Mo}_2\text{O}_5$ . In recent years the method has been investigated by SCHRICKER and DAWSON (112) and by GERRITZ (57), who have modified the reagent in order to be able to use the method also on strongly salt-containing samples.

According to the original description, the reaction is carried out as follows: the sample is mixed with the reagent, after which the whole is diluted with 9 volumes of *boiling* water so that the colour shall reach its maximum. The measuring is performed after cooling as comparative photometry. ZINZADZE states that the colour is very stable (protected from light and air it will keep unchanged for over a week) and is proportional to the phosphorus-concentration within the limits  $4.4 \cdot 10^{-3}$  to  $4.4 \gamma$  phosphorus per ml. The warming by dilution with boiling water is from several points of view scarcely an attractive feature. In a later work, however, also other temperature-conditions are given to allow of full development of colour, namely,  $20-30^\circ$  for three days,  $50^\circ$  for 10 hours,  $70^\circ$  for 3 hours or  $95-100^\circ$  for 30 minutes. It would thus be possible to adapt the method also for the ultra-micro scale.

In the investigation to which I submitted the reaction I first studied the dependence of the colour-development on the temperature and the time when using the original reagent. I here confirmed that for a certain temperature a certain minimum period was required for the colour to reach its maximum. It proved, moreover, that too long a warming leads to a diminishing of the colour, and also that *the colour is not directly proportional to the phosphorus-concentration*. In table 12 are given some values illustrating this. The reaction-periods in the table give the warming periods that result in maximum colour. Shorter or longer periods give lower values for the specific extinction-coefficients.

Table 12.

*Zinzadze's coeruleo-molybdate reaction. Standard phosphate + 0.7 ml of Zinzadze's original reagent (131) + water to 50 ml, heating for a certain period at a definite temperature, cooling, photometry.*

Temperature	60°	70°	80°	90°	100°
Reaction period	2—14	2—6	2—4	1½—2hr	20—60min.
$\gamma$ P/ml	Specific extinction-coefficient				
1.24 . . . . .	395	403	406	403	435
1.86 . . . . .	392	392	400	404	421
2.48 . . . . .	385	384	390	395	416

Next, the relation of the specific extinction-coefficient to the concentration of phosphorus was tested for a greater concentration-interval, and with colour-development at 60° and also at 100°. As is shown in table 13, the specific extinction-coefficient always sinks with rising concentration, i. e. the reaction becomes more and more incomplete. As, however, this diminishing of the specific extinction-coefficient proceeds rectilinearly (figure 4), it is possible to calculate the correct specific extinction-coefficient  $k_c$  for a certain extinction and thus to calculate the concentration. If one plots the specific extinction-coefficient against the extinction, one obviously gets  $k_c = k_0 - a \cdot E$ , where  $k_0$  is the specific extinction-coefficient at the concentration 0,  $E$  the measured extinction and  $a$  the cotangent for the angle between the  $k$ -line and the ordinate (see figure 4).

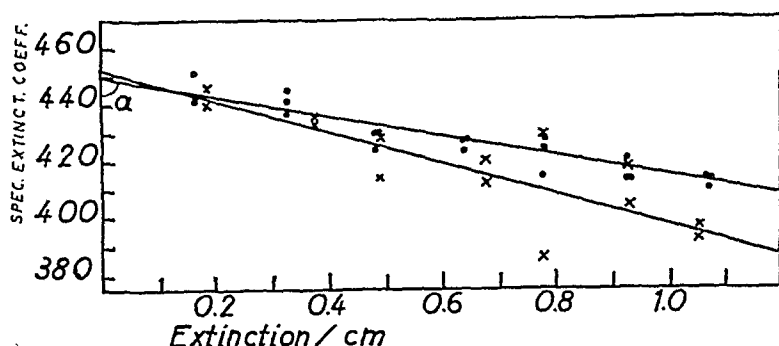


Fig. 4. The decrease of the specific extinction-coefficient with rising extinction in the method of Zinzadze. (—, — colour-development at 60°, —x— colour-development at 100°).



Table 13.

*Zinzadze's reaction. Variation of the specific extinction-coefficient with the concentration of phosphorus. Colour-development at 60° for 6 hours and at 100° for 25 minutes.*

Temp.	60°			100°	
Reagent no.	Z 1.	Z 2.	Z 4.	Z 1.	Z 2.
$\gamma$ P/ml	Specific extinction-coefficient				
0.37 . . . . .	451	441	443	440 446	524 492
0.74 . . . . .	441	437	445	436 433	484 485
1.12 . . . . .	430	424	430	414 428	466 475
1.49 . . . . .	427	427	423	412 420	461 465
1.86 . . . . .	427	414	424	429 385	457 454
2.23 . . . . .	420	413	422	418 404	439 438
2.60 . . . . .	413	410	413	392 396	435 402
$\alpha$ . . . . .	35	35	35	56	68

Despite the more complicated calculation of the analytic result, however, ZINZADZE's method is as regards accuracy comparable with FISKE-SUBBAROW's method, for the dispersion in the specific extinction-coefficient for a certain concentration is of the same order of magnitude as in the latter method. For two different reagents the procentual dispersion in  $k_c$  was for different phosphorus-concentrations between 1.35 and 3 %. One thus seemed to be justified in trying to adapt the method for the ultra-micro scale.

After the investigation of ZINZADZE's original method the composition of the reagent was control-tested, the reagents described by SCHRICKER and DAWSON and by GERRITZ being taken as the points of departure. These authors use stronger sulphuric acid than ZINZADZE, which in certain cases may be an advantage; but they employ a smaller quantity of molybdic acid. As regards the molybdenum pentoxide, they give widely disparate concentrations as the most suitable. As their investigations deal chiefly with strongly salt-containing phosphate so-

lutions, the significance of the concentration of molybdic acid and molybdenum pentoxide on pure phosphate solutions was control-tested with the higher sulphuric acid-content in the reagent as used by these writers. In all the combinations that were tested I found the diminishing of the specific extinction-coefficient with rising phosphate-concentration. Both with the reagent recommended by SCHRICKER and DAWSON and with that recommended by GERRITZ, this diminishing was considerably stronger than was the case with ZINZADZE's original reagent. But by increasing the content of molybdic acid and at the same time so adapting the content of  $\text{Mo}_2\text{O}_5$  that the quotient between the molar concentration of  $\text{MoO}_3$  and the normality of  $\text{Mo}_2\text{O}_5$  (determined by permanganate or cerium titration) was between 1.5 and 2.0, it was possible considerably to reduce the change in specific extinction-coefficient accompanying changes in the phosphorus-content.

The importance of the quotient  $[\text{MoO}_3]/[\text{Mo}_2\text{O}_5]$  emerges from a series of experiments with nine different reagents that were tested with five different phosphate-concentrations. All the nine reagents had the same concentration of sulphuric acid and molybdic acid; only  $[\text{Mo}_2\text{O}_5]$  varied. A résumé of these experiments is given in table 14, from which it will be seen that the factor  $a$  for the calculation of the specific extinction-coefficient (see above) is least for those reagents having the quotients 1.5—2.0.

Table 14.

*Modified Zinzadze's reaction. Final concentration 0.36 n  $\text{H}_2\text{SO}_4$ , 0.0027 mol  $\text{MoO}_3$ ,  $\text{Mo}_2\text{O}_5$  is given in the table, row 2, in m. eq./liter.*

No. of reagent	1	2	3	4	5	6	7	8	9
$\text{Mo}_2\text{O}_5$ . . . . mn	1.01	1.18	1.27	1.35	1.50	1.66	1.86	2.22	2.7
$\frac{\text{MoO}_3}{\text{Mo}_2\text{O}_5}$ . . . . .	2.7	2.3	2.15	2.0	1.8	1.63	1.45	1.22	1.0
$\gamma$ P/ml	Specific extinction-coefficient								
0.37 . . . . .	519	522	529	511	535	552	508	597	682
0.74 . . . . .	475	483	480	476	515	509	499	524	—
1.24 . . . . .	436	464	462	491	469	480	484	493	511
1.86 . . . . .	418	440	459	431	461	496	476	472	478
2.48 . . . . .	407	424	431	427	452	472	467	474	474
$a$ . . . . .	?	85	85	80	75	45	45	?	?

Of these reagents no. 5 was chosen for continued investigation.

It is clearly not possible so to make up the reagent that the reaction will be equally complete for all phosphorus-amounts also within a small concentration-interval. The favourable results with ZINZADZE's original reagent therefore encourage one to prefer it to these others. If, however, it is desired to use a stronger sulphuric acid, it is clear that one should use a reagent that has been made up according to the indications given above. A suitable reagent of this sort is no. 5 in table 14. It is prepared in the following way: 5.7 g of analytically pure  $\text{MoO}_3$  is dissolved in 100 ml of 36 n  $\text{H}_2\text{SO}_4$  (concentrated) during heating. When the solution has been left to cool to  $150\text{--}180^\circ$ , 0.26 g of molybdenum powder is dissolved therein. The reagent when ready for use should contain 36 n  $\text{H}_2\text{SO}_4$ , 0.27 molar  $\text{MoO}_3$  and 0.15 n  $\text{Mo}_2\text{O}_5$ , and it is employed in the proportions of 1 volume of reagent to 100. With this reagent one obtains about as good values as with ZINZADZE's original method, as is seen from table 15. The dispersion in the specific extinction-coefficient for one and the same phosphate-concentration amounts to approximately 1—5 %.

Table 15. 1

*Testing of reagent 5 according to table 14. Final concentrations 0.36 n  $\text{H}_2\text{SO}_4$ , 0.0027 mol  $\text{MoO}_3$ , 0.0015 n  $\text{Mo}_2\text{O}_5$ . The values give the specific extinction-coefficient.*

No. of reagent	Z. V 1.	Z. V 2.	Z. V 3.
0.37 $\gamma$ P/ml . . . .	465	448	500
	441	433	505
	444	421	508
0.74 " . . . .	447	446	494
	445	441	480
	442	422	474
1.24 " . . . .	453	446	485
	426	426	477
	443	419	473
1.86 " . . . .	435	433	474
	424	407	468
	432	413	476
2.48 " . . . .	430	417	456
	419	407	459
	416	402	461
" . . . . .	21	22	40

For the adaptation of either method to the ultra-micro scale the simplest way was first tried, viz. the sample was dissolved in a drop of diluted reagent and the sample-drop put in a micro-cuvette, after which the whole cuvette was warmed for the required time. Even when the colour-development took place at only 50 or 60°, it was impossible to get the hydrophobic layer in the micro-cuvette to hold, so the method was abandoned. Instead, the following technique was tried: after dissolving the sample in the drop of reagent the latter is sucked up in a glass capillary, that is then closed by melting. The colour-development can now take place at 100°, which is often suitable. Preliminary experiments, however, gave very varying results, and one suspected that the relatively large glass surface with which the sample-drop was in contact during the colour-development might have an injurious effect, partly by giving off disturbing substances and partly by diminishing the acidity. In order to investigate the diminishing of acidity the following experiment was performed: on a paraffined surface 7.78  $\mu$ l of sulphuric acid was pipetted with a semi-automatic micro-pipette (see 89). The drop of acid was sucked up carefully in a glass capillary (0.5 mm inner diameter), that was then closed at both ends by melting. After being boiled in water for an hour the capillary was dried and the fluid quantitatively transferred to a 250  $\mu$ l vessel containing 80  $\mu$ l of 0.1 n KIO<sub>3</sub> and 20  $\mu$ l of 0.1 % starch-solution. After the addition of potassium iodide the released iodine was titrated with thiosulphate.

For exact iodimetric micro-acid-determinations a correction is required, as a certain minimum concentration of the acid is needed to release an observable amount of iodine. This correction was determined by the titration of 4 known amounts of sulphuric acid with two different volumes on the same micro-scale as that used in the experiments with the capillaries. From the differences between the different titration-values was calculated the correction: 0.9  $\mu$ l of 0.02 n acid is added per 100  $\mu$ l of final volume. (Table 16.)

As a comparison with the boiling experiments precisely similar experiments were carried out, but sulphuric acid was transferred immediately from the capillary to the micro-vessel. The experiments are reproduced in table 17. They show that the acid-losses on boiling amount to about 0.064 micro-equivalents on an average, with variations of more than 100 % of this value. As the ready diluted sample-solution in ZINZADZE's original method contains 0.25 micro-equivalents of acid per  $\mu$ l, the acid-losses in

Table 16.

*Determination of the volume-correction in connection with micro-iodimetric determination of acid. Titration with 0.0193 n thiosulphate.*

$\mu\text{l}$ 0.020 n $\text{H}_2\text{SO}_4$ :	a) 6.57	b) 13.18	c) 19.75	d) 26.36	
Series I. Final volume approx. 108 $\mu\text{l}$ . . . . (29.12)	Thiosulphate consumption in $\mu\text{l}$				
	5.82	12.60	19.20	25.96	
	5.78	12.68	19.54	26.22	
	5.74	12.60	19.26	26.32	
	5.64	12.50	19.24	26.16	
	5.58	12.76	19.32	26.40	
	5.82	12.60	19.34	26.20	
	5.74	12.52	19.48	25.84	
	5.56	12.74	19.56	25.74	
	5.72	12.46	19.50	26.26	
	5.62	12.42	19.34	26.22	
	5.68	12.72	19.44	26.30	
	5.86	12.60	19.46	26.24	
	Mean values . . . . .	5.71	12.60	19.39	26.16
	Calculated consumption* .	6.78	13.60	20.20	27.10
Difference† . . . . .	+1.07	+1.0	+0.81	+0.94	
Series II. Final volume approx. 212 $\mu\text{l}$ . . . . (30.12)	5.14	11.56	18.66	25.23	
	4.80	11.76	18.54	25.40	
	5.10	11.78	18.66	25.28	
	5.16	11.60	18.70	25.70	
	5.14	11.70	18.36	25.16	
	4.66	11.88	18.58	25.06	
	5.14	11.74	18.58	25.10	
	5.08	11.50		25.00	
		11.80		25.36	
	Mean values . . . . .	5.03	11.70	18.58	25.14
Calculated consumption* .	6.68	13.40	20.04	26.80	
Difference† . . . . .	+1.65	+1.70	+1.46	+1.66	
Difference between groups I and II† . . . . .	0.68	0.90	0.80	1.02	

\* From the differences b—a, c—b and d—c it is calculated that 1.0  $\mu\text{l}$  of  $\text{H}_2\text{SO}_4$  corresponds in series I to 1.033  $\mu\text{l}$  of thiosulphate, in series II to 1.017  $\mu\text{l}$  of thiosulphate. The calculated thiosulphate consumption is based on these values.

† From all the differences the mean correction is calculated as 0.90  $\mu\text{l}$  of 0.02 n acid per 100  $\mu\text{l}$ , that is thus to be added to the titration-value.

Table 17.

*Acid-loss on boiling in glass capillaries.*

Column I: direct pipetting in the test-tube. II: passage via capillary. III: passage via capillary which is then closed by melting and boiled in water for 1 hour. In group A 0.197 n  $H_2SO_4$  was used and the titration was made with 0.1 n thiosulphate. In group B 0.05 n acid and 0.0259 n thiosulphate were used. The titration-volumes are given in micro-liters, and have been corrected for the volume (table 16), that was approx. 170  $\mu$ l.

	I. $Na_2S_2O_3$	II. $Na_2S_2O_3$	Loss through capillary pas- sage micro- equiv. acid	III. $Na_2S_2O_3$	Loss in rela- tion to II in micro-equiv. acid
A.	14.23	13.32	0.086	13.56	0.047
	14.24	14.07	0.011	7.51	(0.652)
	14.21	13.63	0.055	13.76	0.027
	13.98	14.20	0	12.37	0.166
	13.79	14.20	0	13.58	0.045
	13.91	14.03	0.015	13.71	0.032
	14.54	14.34	0	13.48	0.055
	14.53	13.92	0.026	13.31	0.072
	14.20	14.38	0	13.66	0.037
	14.14	14.13	0.005	13.95	0.008
		14.20	0	13.81	0.022
Mean	14.18	14.03	0.018		
B.	13.85	13.44	0.009	7.49	0.152
	13.75	13.08	0.019	10.57	0.072
	13.83	13.47	0.009	8.59	0.124
	13.78	13.30	0.013	12.67	0.018
	13.81	13.65	0.004	10.27	0.08
				10.16	0.083
				9.94	0.089
				10.38	0.077
				11.59	0.046
				10.09	0.085
Mean	13.80	13.39	0.011		0.064

the glass capillaries can explain marked disturbances in the colour-development.

In order to meet these difficulties it was necessary to use glass of the highest quality for the capillaries. The only special glass I have so far been able to test is Jena Geräte glass; but although this resulted in a decided improvement in the values the variations are still so great that the method is of no practical use. Table 18 may serve to illustrate this.

Table 18.

Testing of Zinzadze's method on the ultra-micro scale. A suitable amount of standard  $KH_2PO_4$  solution was left to dry on a glass needle, and was then dissolved in the reagent (1.4 ml of original reagent + water to 100 ml). The sample-drop was sucked into a capillary, that was then closed by melting. Colour-development for 25 minutes at  $100^\circ$ . Photometry in micro-cuvette. The values represent the specific extinction-coefficient  $k$ . Series 1 and 3 were carried out on the same day; series 2 and 4 on another day.

Amount of P in $10^{-3}$ ;	0.82		1.55	
$k$ . . . . .	1) 610	2) 330	3) 457	4) 325
	650	440	565	434
	665	640	385	485
	615	585	445	710
	670	780	505	555
	555	550	406	468
	708	620	485	280
		590	418	340
Mean values	639	564	458	450

Also with the stronger  $H_2SO_4$ -acidified reagent 5 (table 14) the variations were much too considerable. Owing to the present difficulty of procuring special glass it has not been possible to complete the investigation. It may, however, already be asserted that there is little likelihood of this method proving suitable for the ultra-micro scale.

To sum up, it may be said that ZINZADZE's reaction is just as accurate as FISKE-SUBBAROW's and at the same time about three times as sensitive. It is, however, not well-adapted for the ultra-micro scale.

\*            \*            \*

*Denigès' method 1927.* — The method of DENIGÈS of 1927 (49, 50), here called the molybdenum pentoxide method, has been worked out to replace the instable stannous chloride in DENIGÈS' earlier method with a stable reagent. According to DENIGÈS' own description, the reagent consists only of an  $H_2SO_4$ -acidified solution of  $Mo_2O_5$ , that is obtained by the reduction of ammonium-molybdate solution with metallic copper. Actually, a certain amount of  $MoO_3$  is re-formed by the copper catalyzing the air-oxidation of the 5-valent molybdenum in the pentoxide; and DENIGÈS himself remarks that  $MoO_3$  must be present at

the reaction. In the form in which the method is carried out by PEREIRA (102), molybdate solution is added separately, so that the conditions become more well-defined regarding the  $\text{MoO}_3$  surplus. In order that the phosphate, molybdic acid and molybdenum pentoxide in the reaction may unite to form the blue complex, a certain heating is required. According to DENIGÈS, the sample must be boiled, a procedure that is not possible on the ultra-micro scale. When control-testing I have therefore followed PEREIRA's prescription with heating in a boiling water-bath.

According to DENIGÈS, the molybdenum pentoxide reagent is used in such a way that the final concentrations are 0.2—0.4 n sulphuric acid and 0.003—0.006 mol molybdenum. In PEREIRA's procedure one gets the following definitive concentrations:  $\text{H}_2\text{SO}_4$  0.55 n,  $\text{MoO}_3$  0.003 mol and  $\text{Mo}_2\text{O}_5$  0.0011 n. As neither DENIGÈS nor PEREIRA explains the reasons for these concentrations, and as the preliminary test of the two methods did not give the high specific extinction-coefficient stated by PEREIRA, the importance of the variations in the composition of the reaction-mixture when ready for use was investigated.

After preliminary experiments, that showed that an acidity of 0.5 n sulphuric acid is suitable for the reaction, I controlled the significance at this acidity of the relation between the concentrations of molybdic acid and molybdenum pentoxide. The results of this experiment are given in table 19. From this table one

Table 19.

*Testing of Denigès' molybdenum-pentoxide method with 0.5 n  $\text{H}_2\text{SO}_4$  and variation of the quotient  $[\text{MoO}_3]/[\text{Mo}_2\text{O}_5]$ . The concentrations of  $\text{MoO}_3$  are given in mmol and those of  $\text{Mo}_2\text{O}_5$  in milli-equivalents/liter. The table gives the values of the specific extinction-coefficient.*

$[\text{MoO}_3]$ . . . .	0.3	1.1	3.2	1.9	3.4	2.7	3.25	3.35	3.35	3.25
$[\text{Mo}_2\text{O}_5]$ . . . .	2.7	2.7	5	2.7	3.8	2.7	2.95	2.85	1.9	0.95
$\frac{[\text{MoO}_3]}{[\text{Mo}_2\text{O}_5]}$ . . .	0.11	0.41	0.64	0.7	0.84	1.0	1.1	1.18	1.74	3.36
$\rho$ , P/ml										
0.37 . . . . .	110	405	474	420	500	433	514	460	460	325
0.74 . . . . .	108	432	472	460	486	452	435	460	452	337
1.12 . . . . .	103	416	456	456	456	456	438	440	435	332
1.86 . . . . .	107	407	432	445	447	452	445	447	440	290
2.48 . . . . .	101	380	433	447	452	452	445	444	444	278



sees that the specific extinction-coefficient remains on the whole unchanged even if the quotient  $[\text{MoO}_3]/[\text{Mo}_2\text{O}_5]$  varies from 0.6—1.7.

The next thing to be investigated was the significance of variations in acidity at about the same concentrations of molybdic acid and molybdenum pentoxide (quotient about 1). The results, that are given in table 20, show that the sensitiveness for variations in acidity is very slight, as the specific extinction-coefficient is unchanged for acidities between 0.4 and 0.7 n acid. It appears from the two tables that the specific extinction-coefficient has a certain tendency to diminish when the concentration of phosphorus increases. In larger series this emerges still more clearly. It is thus evident that both DENIGÈS' molybdenum pentoxide method and ZINZADZE's method have the same weakness, namely, that the reaction is more incomplete with rising amounts of phosphorus.<sup>8</sup> In DENIGÈS' reaction this decrement in the specific extinction-coefficient is about as great as in ZINZADZE's method; but the sensitiveness for variations in the composition of the reagent seems to be less. The dispersion in the values for  $k$  at a certain phosphorus-concentration is also approximately the same as in ZINZADZE's method.

Table 20.

*Denigès' molybdenum-pentoxide method. The significance of the variations in acidity with the quotient  $[\text{MoO}_3]/[\text{Mo}_2\text{O}_5] = 1$ . The analytic values have been recalculated and expressed as the specific extinction-coefficient. Total molybdenum-concentration = 5.5 mmol.*

Normality $\text{H}_2\text{SO}_4$	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
$\% \text{ P/ml}$								
0.37 . . . . .	243	540	515	487	460	487	473	337
0.74 . . . . .	304	473	486	480	460	487	460	243
1.12 . . . . .	290	462	467	462	452	473	430	300
1.86 . . . . .	332	465	471	460	452	436	394	215
2.48 . . . . .	348	455	448	455	450	395	390	200

As the molybdenum pentoxide method seems to be less sensitive for variations in the reagent, and as a high acidity is favourable for the reaction, it was tested on the ultra-micro scale in spite of the poor results that were obtained with ZINZADZE's reaction.

<sup>8</sup> PEREIRA states that the colour is directly proportional to the phosphorus-content, but I have been unable to verify this.

The test was performed analogously with that of ZINZADZE's method. A series of these experiments is given in table 21. It is evident that the molybdenum pentoxide method according to DENIGÈS is also unsuitable for the ultra-micro scale. Since the acidity in these experiments is considerably higher than in ZINZADZE's reaction and its modification, and since DENIGÈS' method, as is shown in table 20, is not very sensitive for variations in the acidity, the experiment in table 21 shows that it must be impurities that go into solution from the glass that prevent the application of ZINZADZE's and DENIGÈS' methods on the ultra-micro scale.

Table 21.

*Testing of the molybdenum-pentoxide method on the ultra-micro scale. Three different amounts of  $KH_2PO_4$  were dried on glass needles and then dissolved in a reagent containing 0.7 n  $H_2SO_4$ , 0.0028 n  $Mo_2O_5$  and 0.0026 mol  $MoO_3$ . The sample-drops were sucked up in capillaries of Jena Geräte glass, which were closed by melting. Colour-development for 10 minutes at 100°. Micro-photometry. The table gives the values of the specific extinction-coefficient.*

Amount of P in $10^{-3}$ g	0.5	1.0	1.5
	504	309	322
	428	380	380
	1 054	382	405
	120	152	412
	490	480	380
	354	350	350
	282	362	480
	934	550	390
	254	650	330
		620	380
Mean values	490	424	383

To sum up, it may be said that DENIGÈS' molybdenum pentoxide method is comparable with the other methods tested above as regards reproduceability when working with pure solutions. For the ultra-micro scale, however, it is, like the allied reaction according to ZINZADZE, unsuitable.

*Denigès' stannous chloride method* 1920. — OSMOND (100) was probably the first to use the reduction of phospho-molybdic acid with stannous chloride for the photometric determination of phosphorus. His point of departure was the precipitation of phospho-molybdate. But not until DENIGÈS (47) and WU (127) had simultaneously shown that the phospho-molybdic acid in the presence of molybdic acid could be reduced selectively with stannous chloride was it possible to create a modern photometric micro-method. Besides DENIGÈS himself, a large number of writers have made contributions to the technique of the method. Various stannous chloride methods have also been extensively used both in biochemistry and in other fields, especially hydrology and agricultural chemistry.

A survey of some common stannous chloride methods shows that different authors have employed very different amounts of reagent. Especially the concentrations of sulphuric acid and of molybdic acid vary greatly. It has frequently been asserted that a certain concentration of molybdate requires a certain sulphuric acid-content to prevent the excess molybdate from being reduced as well. As a rule, however, each writer has only investigated the significance of the proportions of reagent within a small concentration-interval around the "optimal" strength. A list of some methods is given below.

Author.	Final concentration of		
	H <sub>2</sub> SO <sub>4</sub> n	MoO <sub>3</sub> * mol	SnCl <sub>2</sub> %
ORLE (99) . . . . .	0.135	0.0014	0.0012
RASCHKOWAN (arsenic, 108) .	0.108—0.576	0.0018—0.0096	0.0028—0.0044
ATRINS (5) . . . . .	0.27	0.00285	0.005
KALLE (69) . . . . .	0.9	0.00285	0.003
V. WRANGELL (126) . . . . .	0.22	0.0031	0.0075
TROUGH and MEYER (119) . .	0.40	0.0057	0.005
PARKER and FUDGE (101) . .	0.275	0.0057	—
TROPP and co-workers (118) .	0.525	0.0078	0.02
DENIGÈS (48) . . . . .	0.63	0.010	—
MALJUGIN and co-workers (91)	0.55	0.0114	—
PFEILSTICKER (103) . . . . .	0.28	0.0114	0.0084
BERENBLUM and CHAIN (13) .	1.1	0.0285	—
KUTTNER and co-workers (76, 77) . . . . .	1.1 ± 0.1	0.0306	0.020
TROPP and co-workers (118) .	1.0	0.031	0.04
GOODLOE (61) . . . . .	1.5	0.0365	0.01

\* If ammonium molybdate is given as the reagent the molybdic acid-content has been calculated on the basis of a molybdenum-content of 54.48 % in the salt.

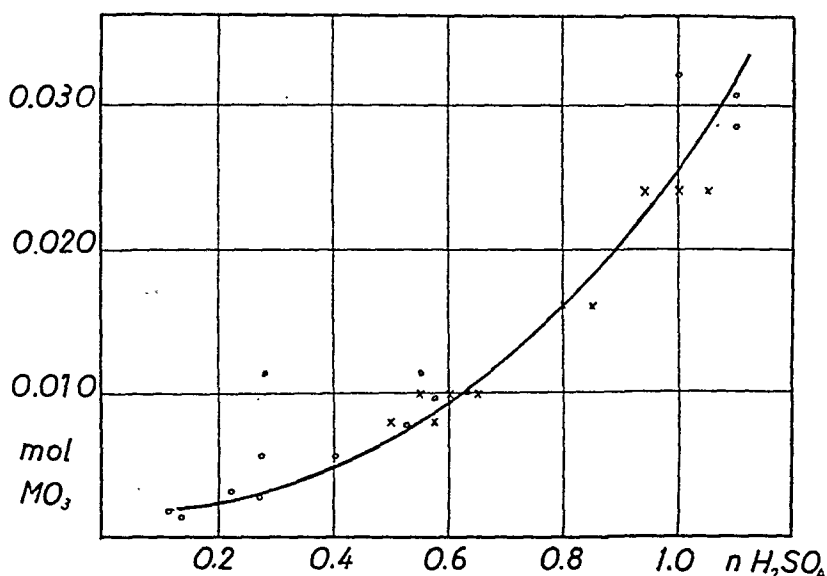


Fig. 5. The correlation between the molar concentration of molybdic acid and the acidity in the stannous chloride coeruleo-molybdate method. (o values from the literature, x values from the author's own experiments).

A graphic representation of the connection between the concentration of the molybdate and the acidity is given in figure 5.

As the recommended concentrations of molybdic acid thus vary greatly, the reaction was first tested with the following concentrations of molybdate: 0.002, 0.004, 0.008, 0.016 and 0.024 mol  $MoO_3$  (as  $Na_2MoO_4$ ) and the sulphuric acid-content was varied. In these tests the concentrations of phosphate were 0.31—1.24  $\gamma$  P/ml. The combinations that gave approximately direct proportionality between the phosphorus-concentration and the colour are marked with a cross in figure 5. For less than 0.008 mol  $MoO_3$ , there was in no case any indication of proportionality within the tested concentration-interval, and the specific extinction-coefficient sank very rapidly with rising phosphorus-content. Nor could variation of the amounts of stannous chloride give any improvement. Experiments show that one can choose the concentration of  $MoO_3$ , rather freely as long as one goes over 0.008 mol and adapts the sulphuric acid-content correctly.

The next test comprised a stricter investigation into the significance of varied amounts of sulphuric acid for a final concentration of 0.010 mol  $MoO_3$ . A summary of a number of these experiments is given in table 22.

Table 22.

Testing of the stannous chloride method with a final concentration of 0.010 mol  $\text{MoO}_3$  (as  $\text{Na}_2\text{MoO}_4$ ). Variation of acidity and stannous chloride concentration. The values give the specific extinction-coefficient.

I. 0.55 n $\text{H}_2\text{SO}_4$ .						
$\gamma$ P/ml	% $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	0.004	0.005	0.006	0.007	
0.12 . . . . .		600		568	581	
0.25 . . . . .		643	648	622	622	
0.37 . . . . .		657	646	641	640	
0.62 . . . . .		689	704	680	671	
0.93 . . . . .		676	664	657	665	
1.24 . . . . .		707	679	656	667	
II. 0.60 n $\text{H}_2\text{SO}_4$ .						
$\gamma$ P/ml	% $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	0.004	0.005	0.006	0.007	0.009
0.12 . . . . .		637	(759)	660	628	635
0.25 . . . . .		640	694	676	634	626
0.37 . . . . .		646	674	655	650	636
0.62 . . . . .		675	717	712	694	657
0.93 . . . . .		675	720	695	665	616
1.24 . . . . .		695	668	717	700	624
III. 0.65 n $\text{H}_2\text{SO}_4$ .						
$\gamma$ P/ml	% $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	0.004	0.005	0.006	0.007	
0.12 . . . . .			(810)	(767)	(781)	
0.25 . . . . .			686	677	669	
0.37 . . . . .			659	657	637	
0.62 . . . . .			692	660	656	
0.93 . . . . .			692	676	673	
1.24 . . . . .			653	623	670	
IV. 0.70 n $\text{H}_2\text{SO}_4$ .						
$\gamma$ P/ml	% $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$		0.005	0.006	0.007	
0.12 . . . . .			586	559	532	
0.25 . . . . .			625	680	586	
0.37 . . . . .			630	614	615	
0.62 . . . . .			692	663	651	
0.93 . . . . .			662	631	654	
1.24 . . . . .			596	595	593	

From this it appears, further, that the acidity is the most important factor in obtaining a proportional reaction, while the stannous chloride may be allowed to vary more without essentially changing the reaction. The best results are evidently obtained with the following *final concentrations*: 0.010 mol  $\text{MoO}_3$  and 0.60 n  $\text{H}_2\text{SO}_4$ .  $\text{SnCl}_2$  may be used in the concentration 0.006 % (about 0.5 mn) but also with up to 0.012 % (about 1 mn  $\text{SnCl}_2$ ) good values are obtained.

Finally, comparative experiments were carried out with these reagent-proportions and those given by KUTTNER and his co-workers, in which connection  $\text{Na}_2\text{MoO}_4$  was tested against  $\text{Am}_2\text{MoO}_4$  as the source of  $\text{MoO}_3$ . These experiments, table 23, show that the reproduceability is good in both cases, with a dispersion of the specific extinction-coefficient of only about 2 %. The specific extinction-coefficient is also slightly different and higher for the lower concentration of molybdate.  $\text{Am}_2\text{MoO}_4$  seems to give a greater dispersion, possibly owing to the formation of more complex ions. In the sequel, therefore,  $\text{Na}_2\text{MoO}_4$  has always been used for the reagent. As regards the reaction-period, special experiments have shown that the colour reaches its maximum within five minutes, and remains thereafter unchanged within the limits of error for at least half an hour. One can thus not agree with BERENBLUM and CHAIN in their assertion (12) that what is gained in sensitiveness is lost in certainty. And it would obviously be an advantage if the stannous chloride method could be adapted to the ultra-micro scale. Attempts to mix, as in FISKE-SUBBAROW's method, all the reagents in *one*, in which the sample could be dissolved, showed that the reagent must then be mixed anew every fourth minute, for such a reagent with stannous chloride "stales" so quickly that already after five minutes it no longer gives a full colour with a phosphate sample. Experiments on the ultra-micro scale were therefore at first arranged in such a way that the sample on the needle was dissolved in a sulphuric acid-molybdate reagent, after which an equal volume of stannous chloride reagent in sulphuric acid was added. Here, too, it proved that the stannous chloride "stales" rapidly. Already 5 or 6 minutes after the mixing of the reagent it was not possible to obtain a complete reduction-effect (colour-strength). But if one used at the most three to four minute-old reagent the method was practicable. As is shown in table 24, the stannous chloride method is as regards reproduceability less cer-

Table 23.

*Comparison between the procedure suggested in these pages for the stannous chloride method and that prescribed by Kuttner and others with 0.030 mol  $\text{MoO}_3$ , and comparison between  $\text{Na}_2\text{MoO}_4$  and  $\text{Am}_2\text{MoO}_4$  as a source of  $\text{MoO}_3$ . The table gives the values of the specific extinction-coefficient.*

[ $\text{MoO}_3$ ] mol	0.010		0.030	
[ $\text{H}_2\text{SO}_4$ ] n	0.60		1.10	
[ $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ ] %	0.006		0.02	
$\text{MoO}_3$ from:	$\text{Na}_2\text{MoO}_4$	$\text{Am}_2\text{MoO}_4$	$\text{Na}_2\text{MoO}_4$	$\text{Am}_2\text{MoO}_4$
$\gamma$ P/ml				
0.124 . . . . .	680	770	608	552
	625	770	608	620
	640	744		552
0.248 . . . . .	650	705	590	578
	645	725	590	602
	645	672	570	584
0.372 . . . . .	620	690	585	576
	650	684	580	593
	660	670	585	589
0.62 . . . . .	630	710	548	605
	630	718	580	605
	620	702	564	690
0.93 . . . . .	628	597	560	640
	628	600	565	635
	645	600	581	658
1.24 . . . . .	635	670	553	614
	650	674	581	625
	635	650	590	650
Mean value	640	686	579	609
$\sigma$ . . . . .	23	55	17	36
$r$ . . . . .	3.6	8	2.9	5.9

tain on the ultra-micro scale than FISKE-SUBBAROW's method (cf. table 9, p. 29). The decisive factor in this connection seems to be the period from the addition of the stannous chloride to the compound reagent till the solution of the sample in the reagent. By defining the times more exactly it would appear not to be out of the question to diminish the error through the colour-development. Apart from this, however, the method is of value

Table 24.

*Application of the stannous chloride method on the ultra-micro scale.  $KH_2PO_4$  that had been dried on a glass needle was dissolved in the stannous chloride compound reagent (with 0.010 mol  $MoO_3$ ) within 3 minutes after the mixing of the reagent. The table gives the calculated specific extinction-coefficients.*

P in $10^{-3} \%$	0.5	1.0
k* . . . . .	915	680
	832	738
	865	661
	822	798
	960	832
	822	950
	705	828
	1 280	748
	995	873
	1 015	918
	1 000	873
	898	918
	1 245	945
Mean value	949	828
$\sigma$ . . . . .	165	97.5
$\sigma'$ . . . . .	17.4 %	11.8 %

\* The values have not been corrected for the blank value.

for the ultra-micro scale, as the sensitiveness is more than four times as great as in FISKE-SUBBAROW's method.

*Summing up*, it may be said that DENIGÈS' stannous chloride method, which is the most sensitive coeruleo-molybdate method for the determination of phosphorus, is under suitable conditions comparable with FISKE-SUBBAROW's method as regards the reproduceability on the micro-scale. On the ultra-micro scale the reproduceability is not so good, but the method can nevertheless be used if a carefully standardized technique with the most scrupulous time-control is observed.

#### *Summary of the experiments with different coeruleo-molybdate reactions.*

A testing on the microgram scale of the four methods that might come into the question for adaptation to the ultra-micro



scale showed all four to be about equally good in respect of reproduceability. Tests on the ultra-micro scale gave the following results:

1. *Fiske-Subbarow's method* gives relatively very good values, and the method can easily be employed for series of determinations (tables 9 and 10, pp. 29 and 30).

2. The considerably more sensitive methods according to *Zinzadze* and *Denigès*, that use a reagent with already reduced molybdic acid, and for which a raised temperature is required for the colour-development, can certainly be technically adapted to the ultra-micro scale, but the reproduceability is so strongly reduced that these methods cannot be considered suitable (table 18, p. 40, table 21, p. 43).

3. *Denigès' stannous chloride method*, finally, which is the most sensitive, can be used on the ultra-micro scale, but the reproduceability easily becomes worse than in *FISKE-SUBBAROW's method* (table 24), which is due to the increasing "inactivation" of the reagent with time. This also makes the more laborious for series of determinations.

As the standard method for the colour-development on the ultra-micro scale, therefore, *FISKE-SUBBAROW's method* has been employed. Also the stannous chloride method has been used for amounts of phosphorus of less than  $10^{-3}\gamma$ .

## CHAPTER 6.

### Mineralization and other preparations for the colour-reaction.

In the previous chapters it has been shown how an extremely small amount of phosphorus as ortho-phosphate can be determined with the aid of a suitable micro-technique and the sensitive coeruleo-molybdate reaction in a suitable form. In order to get all the phosphorus in an isolated, biological material into the form required for the determination it is necessary to carry out mineralization. On the micro-scale this may be done equally well dry or wet (64). In an earlier period the dry mineralization (ashing) was probably most commonly employed, but during the last few decades there is no doubt that wet combustions have been more and more widely used in biochemistry, especially *NEUMANN's*

sulphuric acid-nitric acid method (96) and the perchloric acid method of LEMATTE, BOINOT and KAHANE (79) as well as modifications of these. In this particular case, where the sample is on a fine glass tip (page 4 and fig. 7, p. 55), it is, certainly, not impossible to carry out a wet mineralization with a hanging drop of the digestion-acid; but dry ashing would seem a priori easier to carry out. As after the mineralization the digestion-acid must be totally removed, which may give rise to losses of phosphorus (9), ashing should be even better than wet mineralization. For these reasons I elected to try out in the first place a method for ashing under dry conditions.

Several methods have been described for ashings in connection with the determination of phosphorus. According to HANCE (63), the ashing is performed without additions at 400°. In the majority of methods, however, a surplus of cations is added, as otherwise losses of phosphorus may arise. Thus, TAYLOR and MILLER (115) use sodium carbonate, NEUBAUER (95), PIEN and HERSCHDOERFER (104) and others add calcium acetate, while PIEN and WEISSMAN (105) prefer magnesium acetate. ASHTON (4), ROEPKE (110) and others use magnesium nitrate. GOODLOE (61), finally, recommends zinc oxide. The ashing is generally performed at a faint red glow corresponding to a temperature of 500 to 600°. A closer study of the influence of the temperature seems to have been made only by ASHTON. He states that up to 600° there are no losses, even without addition of cation-surplus (for plants). At 800° there is a loss if magnesium nitrate is not added, and at 1 000° there is a loss even in the presence of magnesium nitrate. TÖRÖK (121) asserts that the phosphates of the alkaline earth metals do not volatilize at the temperature of the Bunsen flame (1870°). BAGINSKI (7) mentions that ortho-phosphates of the alkaline metals give pyrophosphate at 500°, while the calcium-phosphate and the magnesium-phosphate remain unchanged. He does not, however, adduce any analytical evidence for this statement.

In a series of experiments on micro-ashing of samples on a quartz needle in a micro-furnace of clear quartz, where the course of the ashing could be followed through the microscope, a rapid and complete ashing was obtained at 500 to 600°. The yield of phosphate, on the other hand, varied considerably. A stricter investigation of the ashing conditions was therefore carried out.

Into tubes of quartz or Jena Geräte glass were pipetted different amounts of a 10 mmol  $\text{KH}_2\text{PO}_4$ -standard as well as various addi-

tions. This was followed by drying in an air-thermostat at 90—110°, after which ashing for 1 hour was carried out at 500° in a muffle-furnace. After cooling, a number of samples to which had been added excess calcium acetate were dissolved directly in the compound reagent according to FISKE-SUBBAROW, but only between 16 and 45 % of the phosphorus was recovered in this way. As this must be at least partly due to anhydrazation of the ortho-phosphate, the samples were in the sequel always dissolved in 1 n hydrochloric acid, that was evaporated to dryness in the air-thermostat at 100° in order to get a complete hydrolysis of the pyro- or metaphosphate formed.

This was followed by a systematic testing of the significance of a cation-surplus with sodium, calcium and magnesium in the form of acetate. Sodium acetate gave in all the tests too low values. With calcium and magnesium, on the other hand, a suitable surplus gave satisfactory values. A series of experiments with additions of calcium acetate is reproduced in table 25. As appears from this table, it is not possible under the conditions that applied to recover the phosphate completely if no extra cations are added. With a low surplus of calcium, however, *still lower* values were obtained. Only with a big surplus of calcium does one recover the phosphorus more or less completely (95—100 %). A very big calcium surplus, on the other hand, gives a poorer recovery for the bigger quantities of phosphorus. With such quantities of phosphorus it is therefore practical to add such an amount of

Table 25.

*Significance of the cation in incineration of phosphate.  $KH_2PO_4$  + addition incinerated for 1 hour at 500°. Hydrolysis with 1 n HCl. Dissolving in compound reagent according to Fiske-Subbarow's method.*

Multiples of amount of $CaAc_2$ equivalent to the phosphorus	0	2	4	8	12	16	20
Amount of phosphorus in %	Amt. of phosphorus recovered in %						
31 . . . . .	17.8	13.0	30.0	30.3	29.9	29.4	30.7
" . . . . .	17.4	12.9	30.6	29.7	30.2	30.6	32.1
62 . . . . .	26.6	24.3	60.5	59.7	58.3	59.3	59.0
" . . . . .	38.7	24.2	61.0	60.3	59.0	60.0	60.0
93 . . . . .	72.3	54.7	89.5	89.2	86.5	85.0	85.0
" . . . . .	69.6	56.2	90.2	90.0	85.7	84.4	85.0
average % recovered P	62	47	97.6	96.7	94.7	95	94.7

calcium acetate that the surplus for the highest expected phosphorus amount is 4—5 times as much as the amount required for the complete formation of tricalcium phosphate.

In order to show that the losses of phosphorus according to the above account are not due to incomplete hydrolysis of pyro- or metaphosphate, special ashing experiments were carried out. The points of departure for these experiments were potassium pyrophosphate and potassium metaphosphate respectively, with addition of a big surplus of sodium or calcium acetate. A number of such experiments are reproduced in table 26. As is shown by these results, the recovery is good only where calcium has been added. It is therefore probably not the pyro- or metaphosphate formation as such that causes the ashing-losses when the cation surplus is insufficient, but some by-reaction the nature of which has not, however, been made the subject of closer investigation.

Table 26.

*Experiments on the recovery of phosphorus in connection with incineration of pyro- and meta-phosphate with and without cation-surplus.*

Multiples of amount of cations equivalent to the phosphorus		$K_4P_2O_7$		$KPO_3$	
		8 NaAc	8 CaAc <sub>2</sub>	8 NaAc	8 CaAc <sub>2</sub>
Amount of phosphorus in $\gamma$		Recovered phosphorus in $\gamma$			
in $K_4P_2O_7$	in $KPO_3$				
30.6	23.4	16.0	27.8	20.1	20.7
		18.3	29.8	18.1	21.2
58.0	46.0	25.2	56.6	25.2	42.9
		28.4	57.6	27.8	44.4
86.0	66.0	31.2	85.1	46.0	64.5
		40.0	86.1	46.8	63.7

As has already been mentioned, magnesium acetate has the same effect as calcium acetate. To exemplify this there are given in figure 6 some ashing experiments with thymo-nucleic acid (prepared according to HAMMARSTEN, 62) and with casein with in some cases calcium acetate and in other cases magnesium acetate as the addition. In the choice between calcium and magnesium the colour-development is thus decisive. Calcium has the disadvantage that with the sulphuric acid in the reagent it can give rise to difficultly soluble calcium sulphate. With the concentrations of calcium here in question, however, the risk is not so great, and in the actual experiments no clouding by calcium sulphate

was observable. As regards magnesium acetate GOODLOE asserts that it interferes with the colour-reaction. This has not been the case in my experiments on the micro-scale, but in experiments on the ultra-micro scale the colour obtained was in some cases several times as strong as was expected. For this reason the addition of *calcium acetate* was chosen as the standard method for the micro-ashing.

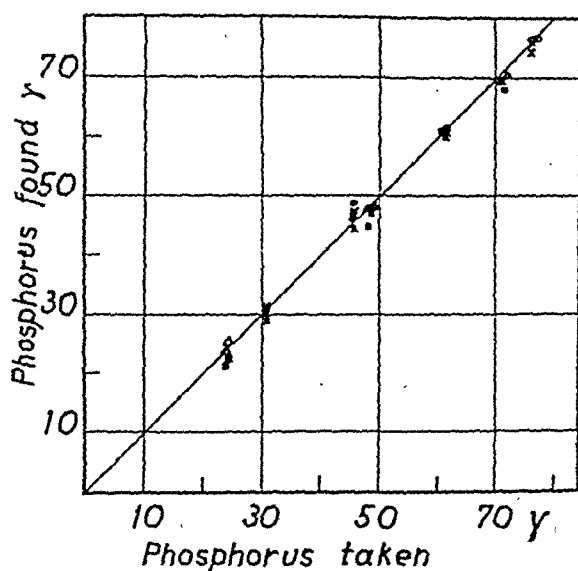


Fig. 6. Recovery of phosphorus after ashing of samples with added calcium-acetate or magnesium-acetate. (o casein plus magnesium-acetate, x casein plus calcium-acetate, ▲ thymonucleic acid plus calcium-acetate, ■ thymonucleic acid plus magnesium-acetate).

The application of the above-described ashing technique on the ultra-micro scale occasioned no difficulties. The problem of carrying out hydrolysis for 1 hour at 100° with 1 n hydrochloric acid and at the same time maintaining the isolation of the sample was solved in the following way. After the ashing, one drop of 1 n hydrochloric acid is pipetted onto each glass needle and the needle is inserted into a closed water-containing chamber at 100°, which is thus saturated with vapour. The drop of hydrochloric acid remains unchanged, or diminishes only slightly in volume in the course of an hour. If suitable, the period for the hydrolysis can be extended even considerably longer. The arrangement is illustrated in figure 7.

In order to control the ashing and the hydrolysis a complete analysis with pure mono-potassium phosphate was performed.

Suitable amounts of standard solutions of  $\text{KH}_2\text{PO}_4$  were pipetted onto quartz needles on which  $1\ \mu\text{l}$  5 millinormal calcium acetate had been allowed to dry. After the samples had dried the needles were placed in suitable holders, which were then placed in a tube-shaped muffle-furnace. When the current had been switched on for 30 minutes the temperature in the furnace had risen to  $500\text{--}525^\circ$ , after which in the course of 2 hours it sank to  $100^\circ$ .<sup>\*</sup> When the holders had been taken out and cooled  $2\ \mu\text{l}$  of 1 n hydrochloric acid was pipetted onto each needle, and the holders were put into the hydrolysis-chamber for an hour. On being taken out, the

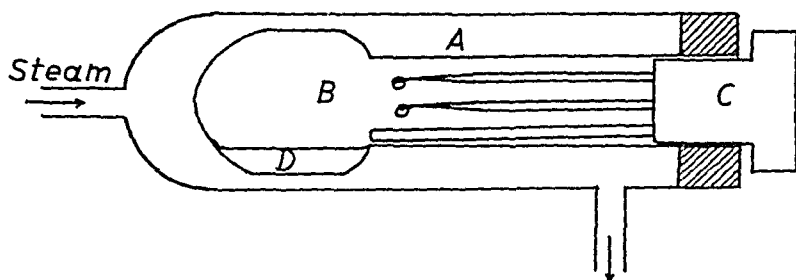


Fig. 7. Apparatus for micro-hydrolysis.

A, steam mantle, B, chamber of hydrolysis. C, holder with quartz needles. D, water.

sample-drops were evaporated at room-temperature. The samples were then dissolved in the compound reagent according to FISKE-SUBBAROW's method, and micro-photometry was performed. The blank-values were determined parallel with the phosphate samples and the appropriate correction was made for all the analytic values. The results of some such experiments are given in table 27.

From this table it appears that the total mean error in a single determination has been more than doubled by the ashing and hydrolysis errors as compared with other errors (table 9, p. 29). Further, these values show a strong, more or less rectilinear diminution in the specific extinction-coefficient with rising amounts of phosphorus. This diminution is found also in experiments carried out with the same technique but with colour-development according to DENIGÈS' stannous chloride method, table 28. As this condition is found in all series after micro-ashing but not with direct phosphate analysis, it must be ascribed either

<sup>\*</sup> The temperature during the ashing was controlled with a thermo-couple continuously during all the experiments. The thermo-couple was calibrated with some fixed temperatures in the usual way.

Table 27.

*Reproduceability in connection with phosphorus-determinations on the ultra-micro scale.  $KH_2PO_4$  with incineration, hydrolysis and colour-development according to Fiske-Subbarow's method. The table gives the specific extinction-coefficients calculated from the analytic values.*

P in $10^{-3}\gamma$	1.0	2.0	2.8	3.8	5.6
k . . . . .	105	119	107	125	103
	190	171	107	118	96
	196	82	175	115	112
	176	128	140	128	91
	134	153	118	134	138
	187	140	175	136	97
	109	155	130	168	128
	154	141	100	141	137
	164	134	154	115	125
	124	170	155	142	125
	109	126	142	129	123
	160		151	137	130
	111		139	92	130
	119		146	144	147
	101		151	151	136
	178		142	163	139
	156		106	160	95
	144		110	162	125
	111			96	117
					118
Mean value	144	146	136	132	121
$\sigma$ . . . . .	32.4	27	23.4	23	16.6
$\nu$ . . . . .	22.5	18.5	17.2	17.5	13.7

to loss of phosphorus on ashing or to the arising of disturbing substances or else to incomplete hydrolysis. The only foreign element that can come from quartz needles is silicic acid, but this is precipitated by the hydrochloric acid on hydrolysis, and can thus not be the cause. The slight amount of calcium that is added can according to experiments on the micro-scale not cause this change. The most probable explanation is therefore that a certain amount of phosphorus is lost on ashing, and in addition to this, some of the phosphorus is not recovered owing to incomplete hydrolysis. Experiments with milder ashing and stronger hydrolysis have not, however, led to any improvement in this respect.

Table 28.

*Reproduceability in connection with phosphorus-determination on the ultra-micro scale.  $KH_2PO_4$  determined after incineration, hydrolysis and colour-development according to the stannous chloride method. The table contains the specific extinction-coefficients  $k$ , calculated from the analytic values.*

Phosphorus in $10^{-3} \gamma$	0.25	0.50	0.75	1.0
$k$ . . . . .	1 070	950	667	735
	1 030	815	630	543
	830	815	771	610
	517	637	660	699
	640	850	645	619
	612	804	665	669
	565	600	610	678
	512	690	605	669
	685	746	705	749
	856	445	590	642
	1 070	547	758	889
	768	815	572	689
		1 010	705	568
			733	
Mean value	765	748	666	675
$\sigma$ . . . . .	209	160	26.8	86.5
$\nu$ . . . . .	27 %	21 %	4 %	13 %

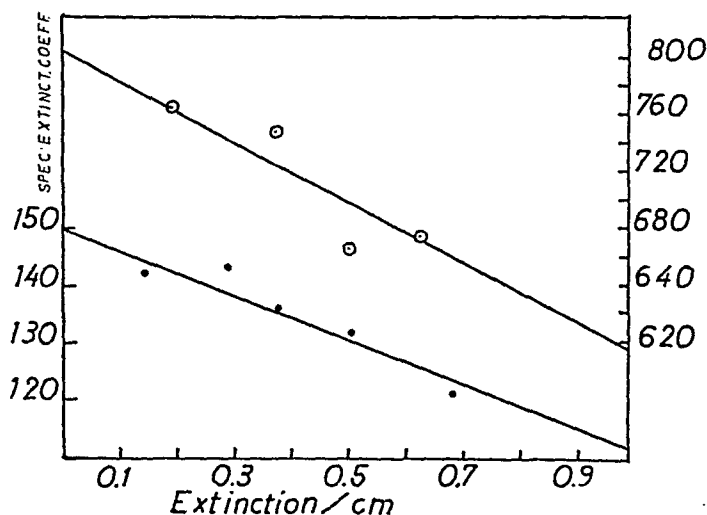


Fig. 8. The decrease of the specific extinction-coefficient with rising extinction on the ultra-micro scale. Method of FISKE and SUBBAROW —●—, ordinates to the left. Stannous chloride method —○—, ordinates on the right side. Reaction volume  $1 \mu l$ .



In order to be able to calculate the amount of phosphorus in a sample it is evident that the specific extinction-coefficient must be determined for the extinction found. This can either be carried out in the same way as has been described for ZINZADZE's reaction (p. 33) or by graphic calculation from figure 8, that has been drawn on the basis of the analyses in tables 27 and 28. Owing to the great analytic error, it is, further, necessary to perform many analyses on individual samples in each kind in order to bring out possible differences on comparing two different objects. The method that has been described in the foregoing is suitable in this respect, as it is well-adapted for series of analyses.

## CHAPTER 7.

### Instructions for the determination of phosphorus on the ultra-micro scale.

In the foregoing chapters the working out of the details for analyses of phosphorus on the ultra-micro scale has been described. For easy reference, short instructions for the different phases as well as a number of operations that precede or follow the various stages of the actual determination are given in the present chapter.

*Preparation of glass.* For the isolation of the samples, fine needles with slightly thickened points are used. The needles are made of quartz or supramax glass (Schott, Jena). The needles are cleansed by boiling in 2 n nitric acid and rinsing in distilled water. Onto the point of each needle is pipetted 1  $\mu$ l of a 5 millinormal solution of  $\text{CaAc}_2$ , in order to ensure a suitable surplus of an alkaline earth-metal ion for the ashing. The importance of the calcium surplus is dealt with in chapter 6.

The object and covering glasses for the photometry are pretreated in the following way: on the thoroughly cleaned glass (alcohol-ether and, if necessary, chromic acid-sulphuric acid mixture) are poured some drops of a solution of 1 g of highly nitrated cellulose (nitrogen-content about 13 %) + 0.1 g of diethyl phthalate + 0.01 g of butyl stearate in 100 ml of butyl acetate. The glasses are placed atilt in special frames and left to dry for at least three days in a place that is free from dust. If a drying drum with an electric fan is used the drying time can be considerably reduced.

When the glasses are dry, two fine lines are drawn with Indian ink over the hydrophobic layer. The distance between the lines should be about 10 mm on the object-glasses and about 4 mm on the covering glasses. So that it may be easier to focus the inked lines sharply in the microscope it is advisable to use diluted ink, when some of the grains will always be so spread out that one can focus on these. The hydrophobic layer is necessary to prevent the drops from spreading out on the glass. If celluloid plates are used instead of an object-glass then no special layer is required, but on the other hand they are not so optically homogeneous.

*Pre-treatment of the samples.* The samples are isolated on the quartz needles according to a method suited to the material (see Part II). If the isolation has been made in water-solution, in which case there is no point in pre-treating the needles with calcium acetate, one pipettes 1  $\mu$ l of 5 millinormal calcium acetate solution onto each needle with sample and leaves to dry in air.

For mineralization the needles are placed in a cold muffle-furnace that is then heated for a certain time (for the furnace I employed, 30 minutes) until the temperature has reached about 500°. The furnace is then allowed to cool with the samples in. Alternatively one may leave the needles in the furnace for 20—30 minutes at a temperature of 500° and then take them out. With all the tested material this method gave complete combustion of the organic substance, so that the sample afterwards contained only inorganic salts. The phosphorus occurs as ortho-phosphate and to a certain extent as pyro- and possibly also meta-phosphate.

Hydrolysis is therefore performed. Onto each needle one pipettes 1 to 2  $\mu$ l of 1 n HCl, after which the samples are placed in the hydrolysis-chamber at 100° for one hour. (Figure 7, p. 55.) If the drop of hydrochloric acid should have evaporated already in the hydrolysis-chamber then the hydrolysis should be repeated. The drops of hydrochloric acid are then evaporated to dryness at room-temperature. All phosphorus should now occur as ortho-phosphate, and the sample is ready for the next step.

*Colour-reaction and photometry.* If the expected phosphorus-amounts are under  $10^{-3}\gamma$  one employs DENIGÈS' stannous chloride method, but with amounts of  $10^{-3}\gamma$  and over it is more suitable to use FISKE-SUBBAROW's method, that is easier to work with. For the first-mentioned method the following reagent is employed: to 10 ml of a solution of 0.010 mol sodium molybdate in 0.60 n sulphuric acid one adds 0.05 ml of a 0.2 n solution of stannous

chloride in concentrated hydrochloric acid (2.5 %  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  gives about this titer). This compound reagent can only be used within the *three* first minutes from the addition of the stannous chloride. The compound *reagent* according to FISKE-SUBBAROW's method is prepared by mixing 24 ml of a solution of 0.022 mol molybdic acid (as ammonium or sodium molybdate) in 0.75 n sulphuric acid with 1 ml of the amino-naphthol-sulphonic acid reagent described on page 27. The mixture is usable for at least an hour.

Immediately before the colour-development the object-glasses are placed on a suitable plane surface with two pieces of covering glasses of about 35 mm thickness along the long-sides. Near one of the inked lines is pipetted a drop for comparison, for which the reagent may be used. In the present investigation, however, I used as a rule 0.75 n sulphuric acid, that reacts in the same way to the hydrophobic layer, while pure water gives a somewhat varying opalescence at the boundary of the layer. The sample is then dissolved in a suitable volume of compound reagent — in general the volumes 0.6 or 1.0  $\mu\text{l}$  have been used, in which connection the little drop is »mixed» on the needle-tip with the point of the pipette for 10 or 15 seconds. During the following 15 seconds the drop is transferred by means of the needle to the object-glass, where it is placed in a row with the comparison-drop at a distance of a few mm, i. e. beside the other inked line. Some drops of liquid paraffin are pipetted around the sample- and comparison-drops, and the covering-glass is laid on in such a way that its inked lines intersect those on the object-glass at four points, between which the drops lie. With a little practice one can carry out the manipulations from the pipetting of the compound reagent to the laying on of the covering glass in 35 or at the most 45 seconds. It is of importance to observe the same conditions for all samples so that the evaporation-effect will be the same (cf. table 3, p. 18).

If the stannous chloride method is employed, the photometry can be carried out already five minutes after the commencement of the colour-reaction, and it should be finished within 45 minutes. In the case of FISKE-SUBBAROW's reaction the samples are left to stand for 30 minutes before photometry is begun. The light-measurement should be finished within two hours from the commencement of the colour-reaction.

If the previously described apparatus (chap. 3) is used for photometry, the amplifier is switched on 30 minutes before the measuring is calculated to begin. The source of light is switched

on five to ten minutes before the measurement. For the actual measurement the object-glass is placed on the measuring stage of the microscope and a part of the sample-drop that is free from dust, that may otherwise cause a serious error in the measurement, is focussed. The displacement that is necessary for the comparison-drop to come into the path of the beam of light instead is noted. The shutter in front of the measuring photo-cell is now opened, and the position of the galvanometer is adjusted with the potentiometer (U in fig. 3, p. 13). When a stable result has been obtained, as a rule after 1 to 2 minutes, the sample-drop is exchanged for the comparison-drop, and the galvanometer-reading is brought back to the previous value by optical compensation with the rotating sector. The sample-drop is now once more focussed, and the initial result controlled. This operation is repeated a couple of times so that accidental errors are eliminated.

*Measurement of the layer-thickness* in the sample-drop is performed in the four points where the inked lines intersect, by sharply focussing the two lines in the microscope (objective 40, num. ap. 0.65) and noting the difference in the number of scale-divisions on the micrometer-screw. As the refraction-index of paraffin-oil is 1.433, the required layer-thickness is found by multiplying the mean value of the number of  $\mu$  according to the micrometer-screw by 1.433.

*Calculation.* The extinction obtained for a sample is multiplied by the inverted value of the layer-thickness in cm, so that the extinction per cm is obtained. From this we subtract the blank-value, that must be determined under the same conditions as the sample, for as compared with the micro-scale the blank-value obtained on the ultra-micro scale is both greater and more varying. The next step is to seek the phosphorus amount corresponding to the corrected extinction per cm on a curve over the connection between the extinction per cm and the phosphorus amount, that was drawn on the basis of analyses of known phosphorus amounts under the same conditions as those obtaining for the investigation of the sample. Alternatively, of course, the specific extinction-coefficient corresponding to the extinction per cm obtained can be determined from a curve (see figure 8) or according to the procedure described on p. 33. The extinction per cm multiplied by the sample-volume in  $\mu$ l and divided by the specific extinction-coefficient then gives the amount of phosphorus in the sample in micrograms.

The method permits of the determination of amounts of phosphorus down to  $5 \cdot 10^{-4}\gamma$  with an error that for the single analysis does not exceed about 20 %. By using a smaller volume than one  $\mu\text{l}$  one can get down as far as  $10^{-4}\gamma$ , but the error may in this case be rather bigger. As a further illustration of the accuracy of the method, some experiments with phosphorus-determination in casein- and thymo-nucleic acid solutions are given in table 29.

Table 29.

*Recovery of phosphorus from casein and thymo-nucleic acid on the ultra-micro scale. Colour-reaction: Fiske-Subbarow's method.*

<i>Casein</i>				
Given amount of P in $10^{-3}\gamma$	1.98		3.96	
Amt. of P recovered	in $10^{-3}\gamma$	in %	in $10^{-3}\gamma$	in %
	1.84	93	3.92	99
	2.44	125	3.23	82
	2.10	106	4.13	104
	2.13	108	3.74	94
	2.08	105	4.09	103
	2.22	112	4.26	108
	2.15	109	3.96	98
	2.02	102	3.74	94
	1.99	100	4.11	104
Mean value		106		98
<i>Thymo-nucleic acid</i>				
Given amount of P in $10^{-3}\gamma$	2.02		4.05	
Amount of P recovered	in $10^{-3}\gamma$	in %	in $10^{-3}\gamma$	in %
	2.27	112	3.90	96
	2.28	113	3.89	96
	1.84	91	4.07	100
	2.02	100	3.94	97
	2.26	112	4.12	101
	2.03	101	4.14	102
	2.17	107	4.14	102
	2.12	105	4.18	103
	2.15	106	3.78	93
	2.00	99	4.00	99
	1.98	98	4.00	99
	2.06	102	4.15	102
Mean value		$104 \pm 6.6$		$99 \pm 3.2$

## PART II.

### Histo- and cyto-chemical determinations of some phosphorus-fractions.

#### CHAPTER 8.

#### The technique for the defining, isolation and fractioning of samples.

In the previous chapter we have seen how phosphorus amounts in organic material can be determined down to  $10^{-4}\%$ . But before such a determination on biological material can have any value it must be possible to define the analysed sample with sufficient accuracy.

It is of course best if the samples can be defined and analysed without the necessity for their being *moved* between these phases. This is the case in the histo- and cyto-chemical investigations that were carried out by CASPERSSON and co-workers, where the definition by micro-photography could also be performed after the analysis (33) or be combined with the analysis (19). In analyses that necessitate ashing of the sample it must be transferred, after micro-photography, if the latter is to be performed, to an ashing needle, since ashing of the sample on an object-glass and dissolving of the ash in a colour-reagent cannot be carried out on the ultra-micro scale because the drop of reagent spreads out over the whole glass and one cannot get the sample in a little volume, which according to chapter 2 is an essential condition.

Another possibility that has been tried is to put the sample in a drop of digestion-solution that floats in an indifferent medium. The sample is defined by micro-photography in the digestion-solution. This technique has not, however, proved successful, as it has been impossible to prevent the drop with the sample from flowing away, on heating, to some glass surface where it immediately spreads out.

*The methods for defining and isolating material for the determination of phosphorus on the micro-scale may be divided into two main groups:*

I. The analysis deals with a natural entity such as a whole egg or a whole organism (protozoon). The natural definition is as a rule sufficient, but if one wishes more closely to define the volume this may be done by sucking up in a calibrated capillary or by micro-photography.

II. In all other cases the part that is to be analysed must be isolated either before or during the characterization. Three principles must here be observed. a) From a suitably fixed organism or from a living, unfixed organism one dissects out or otherwise separates (centrifugation) the part that is to be analysed. The volume of the sample may be defined by sucking up in a calibrated capillary, and for the rest of the characterization micro-photography may be resorted to. b) If two dimensions are known and the isolation takes place along the third by section with a microtome, then the volume is determined already in the act of isolation. This method has been specially developed and studied in the Carlsberg Laboratory, and is well-adapted for series of analyses. The histological definition may then be made direct on the sample, but it is generally made in the preceding and following sections (LINDERSTRØM-LANG and MOGENSEN, 87). c) The isolation is performed on histological sections, one dimension being already known. The isolation is carried out by dissection — for example with micro-manipulator — and the other dimensions and the histological definition are obtained by microscopic observation or micro-photography.

According to the above procedure, the phosphorus-content will be given in weight per volume-unit, e. g. in  $\gamma/\mu\text{l}$  instead of  $\gamma/100\gamma$  (per cent), which is otherwise usually the case. For a conversion to per cent one needs to have a knowledge of the specific weight of the sample and of any shrinking that may occur through the fixation etc. As the specific weight varies quite a lot, even for the same organ, AGDUHR (1, 2), and as the shrinking through the fixation etc. may also vary, this conversion gives only approximate values. For an average shrinking of 10 % and a specific weight of between 1.02 and 1.10 (1, 2) the conversion-factor is 0.0883—0.0818.

All the above-described procedures for the taking of samples have been tried out and have proved to be practicable also for

histo- and cyto-chemical investigations of phosphorus. Analyses of single freely occurring cells have been performed on the eggs of sea-urchins (chap. 12). Isolation by micro-dissection according to II a) has been tried on chromosomes from salivary glands of *Chironomus* (chap. 13). The isolation of samples according to II b) has been used for investigations on the root-tips of onions (chap. 9). A more detailed description of the technique for the isolation by dissection of histological sections, II c), that was used for the remaining material (chaps. 10 and 11), is given below.



Fig. 9. An island of Langerhans has been selected. Enlarged 104 times.

The material is fixed by some suitable procedure and bedded in paraffin. Sections of suitable thickness are then cut from the paraffin block, for example  $10\ \mu$ . The sections are stretched by careful warming on glasses that are covered with a thin film of collodium. The adhesive used is white-of-egg mixture. By immersion in chloroform for 10 minutes the paraffin is removed. After drying the preparation is moistened with paraffin-oil or glycerin, and is now ready for micro-dissection. One next looks through the microscope for the part that is to be isolated, e. g. a Langerhans' island (see figure 9). One then makes four cuts around the desired part with a dissection-needle, cutting also through the collodium film (figure 10). The next step is to cut away and lift off with the dissection-needle the surrounding tissue and collodium film, so that the isolated preparation lies free.

By micro-photography one now determines the nature of the isolated piece of tissue (cf. figure 15, p. 77 and figure 16, p. 83) and also its dimensions. The sample is now to be transferred to





Fig. 10. Four cuts have been made round the island. Enlarged 323 times.

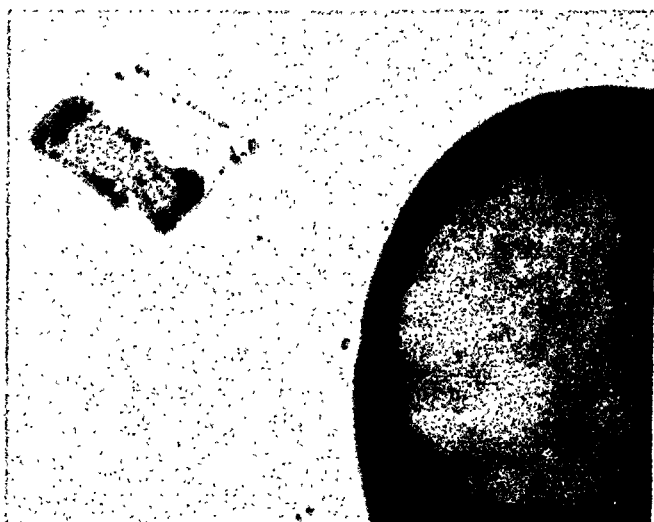


Fig. 11. The needle to which the sample is to be transferred has been placed with its club-shaped tip close to the sample, which has been isolated from the surrounding tissue. Enlarged 104 times.

a quartz needle for the subsequent treatment. The needle is held near the sample, figure 11, which is then lifted towards the quartz needle with the dissection-needle. Thanks to the paraffin-oil the section easily sticks to the needle, figure 12. The sample is now treated further according to the procedure described in chapter 7. A record of analyses illustrating the analytic operations and cal-

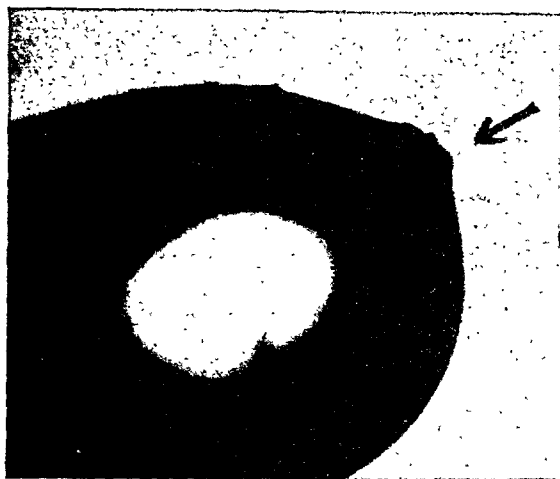


Fig. 12. With a dissection needle the sample has been carried to the quartz needle, where it appears as a contour change. Enlarged 104 times.

Fig. 9—12. The isolation of samples from histological sections. Micro-photos at 275  $m\mu$ .

culations is given in table 34, page 78, with the figures 14 and 15 belonging thereto.

*Fractioning.* The biologically occurring phosphorus compounds may be divided into the following three main groups:

1. Acid-soluble phosphorus = phosphorus-fractions comprising inorganic phosphorus (direct phosphorus, 117), and soluble in 5 per cent trichlor-acetic acid simple nucleotides, carbo-hydrate-phosphoric acid-esters, phosphagenes and some other fractions.

2. Phosphatide-phosphorus = all phosphorus that can be extracted with lipid solvents such as alcohol-ether or alcohol-chloroform from the coagulated residue after the trichlor-acetic acid treatment. As the name implies, this fraction comprises the phosphorus of the phosphatides.

3. Residual phosphorus = the phosphorus remaining after the trichlor-acetic acid and lipid solvent treatment, for the most part nucleic acid phosphorus. Phosphorus from possible phospho-proteins is also included in this fraction, but as the phospho-protein phosphorus constitutes as a rule 0—2 % of the total phosphorus (PLIMMER and KAYA, 106) a division into nucleic acid phosphorus and phospho-protein phosphorus is not called for in the light of the analytic errors on the ultra-micro scale.

Theoretically, the fractioning on the ultra-micro scale can be performed in two ways.

A. The *isolated* sample is extracted so that one gets the fractions from one and the same object. Some experiments according to this principle are given in chapter 9.

B. The extractions are performed *before the isolation*, so that the extracted fractions cannot be determined on the same material (section), but only as difference from parallel samples that have not been extracted. This method has been employed in the histochemical investigations on paraffin-sections (chaps. 10 and 11), where the paraffin and with this also the phosphatides have been extracted (chap. 10). The fractions that it has been possible to determine are therefore the acid-soluble phosphorus and the residual phosphorus.

## CHAPTER 9.

### Experiments with roots of onion, *Allium cepa*.

In investigations on the ultra-violet absorption at 257  $m\mu$  in root-tips of onion, CASPERSSON and SCHULTZ (27) found that this absorption was greatest in the growing zone of the tip, i. e. about the first 2—3 mm, after which it diminished. By means of Bial's tests these authors were also able to show pentoses within the growing zone but not, on the other hand, further up the root. This is very strong support for the assumption that the ultra-violet absorption is here conditioned by nucleic acids, to which CASPERSSON ascribes decisive importance for the formation of protein (37). The final proof of an increased nucleic acid-content within the growing zone would be constituted by the demonstration of an increased content of residual phosphorus. Investigations on the relations of the phosphorus-fractions in the root-tips of onions have therefore been carried out both on the ultra-micro scale and on the microgram scale, thus giving a certain control of the ultra-micro method.

The technique for experiments on the ultra-micro scale is as follows:

A root-tip of about 10 mm in length is cut off, rinsed in distilled water and placed on filter-paper in a little Petri dish, that is then put in the ice-pan of a refrigerator (temperature about  $-20^{\circ}$ ). The refrigerator is specially constructed, so that all further treatment of the root-tip, even including the isola-

tion of the sample, can be performed therein at an air-temperature of about  $-10^{\circ}$ .<sup>10</sup> In principle the construction conforms to that of the freezing technique chest constructed in the Carlsberg Laboratory (LINDERSTRØM-LANG and MOGENSEN, 87). After half an hour the frozen root-tip is taken with a pair of tweezers and held against the object-table of a Minot microtome. A drop of distilled water is placed at the base of the root-tip with a special "pipette". After a second or so the water-drop is frozen. By freezing several drops of water onto the root-tip at intervals of a minute between each drop the whole may be covered with an icicle. After an interval of half an hour, to allow the temperature of the icicle to become uniform throughout, the work of sectioning is begun. Each section curls up on the edge of the knife and may be taken on a quartz needle by means of a slight pressure against the roll. A magnifying glass is used to ensure that the whole roll is taken onto the needle. Otherwise the sample is rejected. In order that the section may not fall from the needle-tip the needle is held over a little platinum wire that is made to glow for a moment with an electric current. The ice in the section then melts to a tiny drop of water around the sample, that then quickly freezes again to ice. When the needles have been taken out of the refrigerator, possible fractioning and the further treatment according to chapter 7 is carried out.

This method was first tried in the determination of the total phosphorus-content. Onions were made to grow in tap-water at

Table 30.

*Determination of total phosphorus in sections of fresh, 8 day-old onion-root, prepared by the freezing technique; diameter 0.5 mm. Amounts of phosphorus in  $10^{-3}\gamma$  per  $1 \mu$  section. Ultra-microscale.*

Distance from tip in mm.	0.75	1.3	1.9	2.9	3.5	4.15
Phosphorus in $10^{-3}\gamma$ . . . . .	1.15	1.62	1.39	1.02	0.64	0.52
	1.44	1.3	1.43	0.9	0.63	0.34
	1.1	1.8	1.3	0.89	0.43	0.38
	1.26	—	1.03	0.62	0.47	0.32
	1.12	—	0.66	0.77	0.46	0.40
Mean value . . . . .	1.21	1.57	1.16	0.84	0.53	0.39

<sup>10</sup> For their kindness in connection with the construction and manufacture of the refrigerator I would like to express my thanks to M. BLONQUIST Esq., civil engineer, and to A. B. Electrolux, Stockholm.

room-temperature. Within one or two weeks 2—5 cm. long root-fibres were obtained, of a thickness ranging from 0.5 to more than 1 mm. An experiment with such a root is given in table 30. The sample-sections were five, seven or ten  $\mu$  in thickness, so the analysed phosphorus amounts were in the region of about 5—10  $\cdot 10^{-3}\gamma$ . All the values in the table have been converted to a section-thickness of one  $\mu$ . The variation-coefficient for such an analysis has not been determined, but it is probably about 15 % (cf. tables 27 and 29, pp. 56 and 62. The error in connection with the preparation of sections by the freezing technique is  $\pm 0.5 \mu$ ). The experiment thus shows a distinct peak on the total phosphorus-curve that falls within the growing zone, figure 13. This agrees well with CASPERSSON and SCHULTZ's experiments (loc. cit.).

In order to investigate the different fractions' respective shares of total phosphorus, fractioning on the ultra-micro scale was also tried. The technique is as follows: After isolating the samples from the quartz needles the latter are taken out of the refrigerator. One or two  $\mu$ l of ice-cold 5 % trichlor-acetic acid is pipetted onto each tip. After 30 minutes the trichlor-acetic acid extract is sucked up with a pipette, on the tip of which a little piece of filter-paper has been sucked fast. The trichlor-acetic acid extract is transferred to a clean needle, dried and then treated according to chapter 7 for the determination of the acid-soluble phosphorus. On the first needle is the section containing the phosphatide and residual phosphorus. Separation of these by extraction of the phosphatides in hanging drop can, certainly, be carried out, but the number of samples that miscarry is a fairly high percentage, so only a small number of such experiments were made. Instead, the above-described fractioning with trichlor-acetic acid was performed on onion-root, that after drying by freezing (58) had been extracted with alcohol-chloroform (98). In order to make sure that the sum of the fractions was equal to the whole, total phosphorus-determination was performed on every other section. Some fractioning experiments according to these two methods are given in table 31. As may be seen from the table, despite the further sources of error entailed by the fractioning, the sum of the fractions agrees in the main with the directly determined total phosphorus. In the experiment with fresh root, table 31 A, it appears clearly that the increased content of total phosphorus in the growing zone is chiefly

Table 31.

*Fractionating of onion-roots on the ultra-micro scale. All the phosphorus-values in  $10^{-3}\gamma$  per section of 1  $\mu$ .*

*A. Fresh, 0.5 mm thick root, 14 days old.*

Distance from tip in mm.	1.2—1.4				3.0—3.15			
Fraction	1.	2.	3.	4.	1.	2.	3.	4.
	Acid soluble P	Residual P	$\Sigma(1+2)$	Total P according to analysis	Acid soluble P	Residual P	$\Sigma(1+2)$	Total P according to analysis
P in $10^{-3}\gamma$ . . .	0.35	0.71	1.06	0.9	0.17	0.11	0.28	0.22
	0.35	0.63	0.98	0.82	0.24	0.15	0.39	0.23
	0.31	0.44	0.75	0.84	0.13	0.17	0.30	0.35
	0.32	0.36	0.68	—	0.16	0.18	0.34	—
	0.15	0.51	0.66	—	0.20	0.24	0.44	—
Mean value . . .	0.30	0.53	0.83	0.85	0.18	0.17	0.35	0.27

*B. 1 mm. thick root, dried by freezing and fat-extracted; 8 days old.*

Distance from tip in mm	1.1				3.0			
Fraction	1.	2.	3.	4.	1.	2.	3.	4.
P in $10^{-3}\gamma$ . . .	2.9	2.9	5.8	4.6	1.5	3.6	5.1	4.7
	2.9	5.9	8.8	7.9	1.4	3.9	5.3	7.6
	2.9	7.0	9.9	6.7	1.1	4.5	5.6	5.1
	2.7	2.0	4.7	11.8	1.6	4.6	6.2	7.5
	3.0	2.0	5.0	11.8	1.1	3.7	4.8	6.7
	3.0	6.5	9.5	9.0				
	1.1	—	—	5.7				
Mean value . . .	2.6	4.4	7.3	8.2	1.3	4.1	5.4	6.3

due to an increase of the residual and phosphatide fractions. Further, it appears from the experiment with fat-extracted root that has been dried by freezing that the phosphatide phosphorus is low in comparison with the residual phosphorus, for otherwise the acid-soluble fraction would constitute a higher percentage in this series than in the experiment in which the phosphatides had not been extracted. A certain reservation should, however, be made, as the extraction with trichlor-acetic acid is possibly

less complete *after* phosphatide-extraction than before, as EULER and SCHMIDT (52) found in connection with the fractioning of dry powder from the testicles of fish. The experiments probably justify the conclusion, however, that the essential increase in the total phosphorus-content in the growing zone as compared with the full-grown part of the root is due to an increase in the residual phosphorus-fraction, i. e. in the nucleic acid-content.

As a check on the ultra-micro method the following experiment was performed on the micro-scale: between 50 and 100 stout onion-root tips (average diameter about 1 mm) of 10 mm in length were placed together with the tips against an upright plane surface, and the whole bunch was left to stiffen in the refrigerator. The bunch of roots was then frozen fast on the object-table of the microtome in the same way as described above for single onion-root tips. This was followed by sectioning in 10  $\mu$  sections. One hundred or more sections were successively transferred to a small test-tube, and 2 ml of 10 % trichlor-acetic acid was added. The suspension thus obtained was then left to stand for half an hour to an hour at + 2°. This was followed by filtration through small ash-free filters and quantitative transference of the remaining precipitate to the filters with 2 % trichlor-

Table 32.

*Fractionating of onion-root tips on the micro-scale. A 10 days old; B 14 days old. Mean diameter 1 mm. per root. Values are given in  $10^{-3}\gamma\text{P}$  for one root in 1  $\mu$  thick section. The stannous chloride method for the determination of phosphorus.*

Mean distance from tip in mm.	Acid soluble P	Phosphat- ide P	Residual P	Total P
A. 0.75 . . . . .	1.44	0.97	3.32	5.73
2.0 . . . . .	2.25	1.35	3.74	7.34
3.25 . . . . .	1.59	0.77	1.74	4.10
4.75 . . . . .	1.48	0.60	1.11	3.19
6.25 . . . . .	1.53	0.50	0.94	2.97
7.75 . . . . .	1.47	0.47	0.80	2.74
9.25 . . . . .	1.09	0.36	0.84	2.29
B. 1.0 . . . . .	0.84	0.63	1.6	3.07
1.6 . . . . .	0.77	0.54	0.95	2.26
2.6 . . . . .	0.73	0.37	0.62	1.72
3.4 . . . . .	0.64	0.31	0.52	1.47
4.9 . . . . .	0.81	0.25	0.39	1.45

acetic acid. The total acid-soluble phosphorus was determined in the filtrate. The filter with the precipitate was dried in vacuum and the lipoids were extracted with alcohol-chloroform in a micro-Soxhlet's apparatus (NORBERG and TEORELL, 97, NORBERG, 98). The phosphatide phosphorus was determined in the extract and the residual phosphorus in the residue after mineralization. All mineralization was performed with sulphuric acid and nitric acid (see TEORELL and NORBERG, 117). On account of the small amounts of phosphorus the stannous chloride method was used for the determination. The phosphorus-values obtained are given in table 32, where all values have been converted to one  $1\mu$  section of one root. The total phosphorus and residual phosphorus values are given also in figure 13.

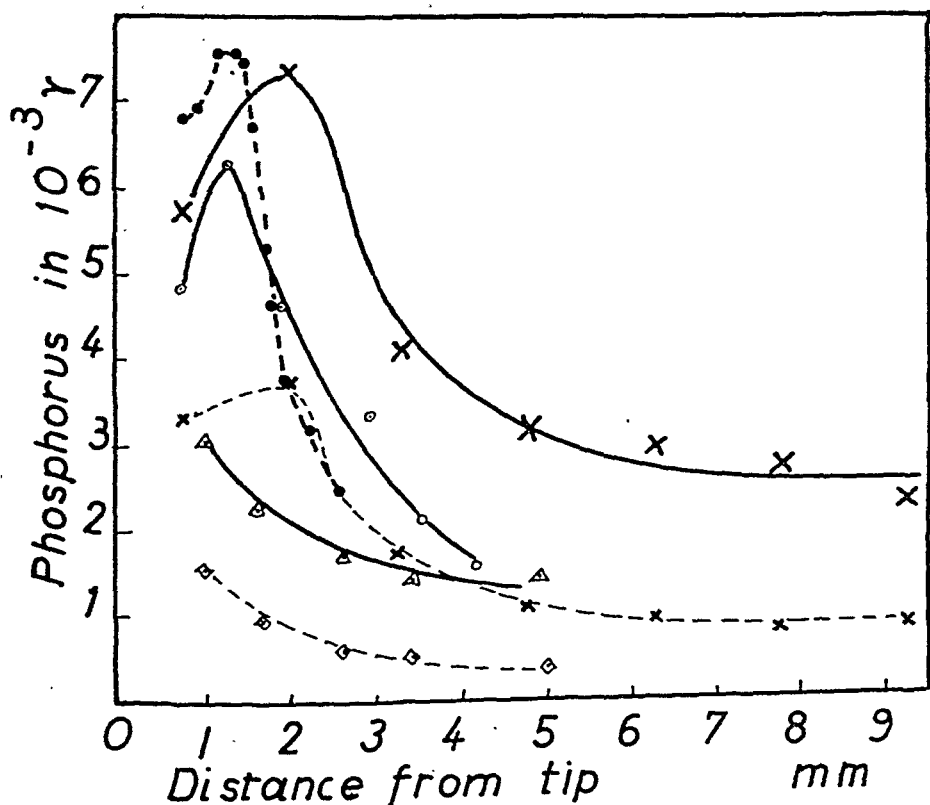


Fig. 13. The phosphorus-content in one  $\mu$  cuts from roots of *Allium cepa* —○— total phosphorus, table 30, values multiplied by four (see text). —●— extinction at 275  $m\mu$  (arbitrary scale) [from measurements by CASPERSSON and SCHULTZ (unpublished result)]. The steeper decrease in the absorption curve as compared with the phosphorus curve is artificial owing to the measuring technique.

—x— total phosphorus, table 32 A. ---x--- residual phosphorus, table 32 A.  
—△— total phosphorus, table 32 B. ---◇--- residual phosphorus, table 32 B.



Experiments on the micro-scale fully confirm the results that have been obtained with the ultra-micro method, namely, that the high phosphorus-content in the growing zone is in a large measure due to residual phosphorus (nucleic acid), in full agreement with the absorption in ultra-violet light at  $257\text{ m}\mu$  and the pentose-content (CASPERSSON and SCHULTZ, loc. cit.), and further, that the content of phosphatide phosphorus is low in relation to the nucleic acid phosphorus, though also the phosphatide-content is higher in the growing zone than in the proximal part of the root. The acid-soluble phosphorus is more evenly distributed over the whole root.

As regards the separate values it may be said that the ultra-micro method gives a more detailed and exact picture of the distribution of phosphorus in the root, while the microgram method only gives the mean value from a large portion of several roots, where also individual variations must play a part. Taking this into consideration, the absolute values according to the micro-method agree well with the values according to the ultra-micro method, fig. 13. The values in tables 30 and 31 A refer to a  $0.5\text{ mm}$  thick root, while the values in table 32 refer to a root of about  $1\text{ mm}$  in thickness. For comparison the former should thus be multiplied by four in order to refer to the same volume, and this has accordingly been done in figure 13. The values according to table 31 B refer, certainly, to a root of  $1\text{ mm}$  in thickness, but as the freezing-drying gave rise to a perceptible shrinking of the root these values should be reduced if they are to be compared with the others. The marked shrinking in connection with the freezing-drying renders this method of fixation unsuitable for this material.

## CHAPTER 10.

### **The phosphorus-content in the endocrine and exocrine parts of the pancreas.**

Even with the ordinary histological staining methods the microscopic picture of the pancreas shows a marked difference between endocrine and exocrine tissue. As CASPERSSON, LANDSTRÖM-HYDÉN and AQUILONIUS (40) have shown, the light-absorption in ultra-violet at  $257\text{ m}\mu$  is considerably weaker in the

islands of Langerhans than in the exocrine part surrounding them. According to the above writers, this is due to the fact that the zymogenous cells contain a large amount of cytoplasmatic nucleotides, that are, on the other hand, almost altogether absent from the insular cells. As it had previously been impossible to perform comparative analyses of phosphorus in the insular organ and the exocrine part of the pancreas from the same animal, some such determinations were carried out both on the pancreas of monkfish, *Lophius piscatorius*, and on the pancreas of the mouse.

*Pancreas of monkfish.* — In the monkfish the endocrine part of the pancreas, the islands of Langerhans, is joined to a special organ, around which lie the small exocrine parts. It has thus been possible to carry out certain macro-chemical analyses on the insular organ (JORPES, 68), which is of interest for comparison with the ultra-micro scale. For the ultra-micro analyses the pancreas of the monkfish was fixed by the freezing-drying method according to GERSH (58), and then bedded in paraffin. 10  $\mu$  thick sections were placed on quartz glass with collodium film. After dissolving the paraffin with chloroform for 10 minutes the sections were moistened with paraffin-oil. Samples were dissected out under the microscope in ultra-violet light (275  $m\mu$ , Köhler's illumination apparatus) from both endocrine and exocrine parts, which were easily distinguishable by their different light-absorptions (40). The results may be seen in table 33 A.

In order to ascertain whether the phosphatides had been completely extracted with the short chloroform treatment of the sections, the preparation was extracted in the micro-Soxhlet's apparatus with alcohol-chloroform (97, 98) for 8 hours. The preparation was then bedded again in paraffin, sectioned and examined anew as above. The results are given in table 33 B. Both for the endocrine and the exocrine parts the mean values for the two series lie within the limits of error for the method. It is thus clear that 10 minutes' extraction with cold chloroform on 10  $\mu$  sections gives a complete extraction of the phosphatides. As according to JORPES' analyses the phosphatide-phosphorus constitutes a third part of the total phosphorus in the insular organ of the monkfish, an incomplete extraction of the lipoids ought to be noticeable even on the ultra-micro scale, despite the greater sources of error. Experiments thus show that the phos-

Table 33.

*The phosphorus-content in the exocrine and insular parts of pancreas of monk-fisk. All the values represent the phosphorus-content in  $\gamma/\mu\text{l}$ .*

	A. Not special fat-extraction		B. Thorough lipoid-extraction before the isolation of the sample	
	Insular part	Exocrine part	Insular part	Exocrine part
	0.92	4.78	0.66	2.24
	1.10	2.90	0.72	3.45
	1.21	3.92	1.73	7.4
	1.70	1.55	0.89	3.75
	1.82	2.74	0.91	1.09
	1.55		1.31	
	2.25		1.87	
	2.30		1.86	
			0.36	
			1.95	
			1.05	
			1.75	
			2.68	
			3.08	
			2.0	
			1.28	
			0.96	
Mean . . .	1.61	3.18	1.47	3.59
$\sigma$ . . . . .			0.73	
$\epsilon(M)$ . . . . .			0.18	

phatide-phosphorus fraction cannot be determined on the ultra-micro scale even after drying by freezing. It is evident that only fresh material is suitable for phosphatide studies, after isolation by for example the freezing technique.

The phosphorus determined in these analyses constitutes the sum of the residual and the acid-soluble phosphorus. The relative values with approximately double the phosphorus-content in the exocrine as compared with the insular part agree with the light-absorption in ultra-violet light, which shows that the poly-nucleotide-content in the exocrine part is considerably greater than in the endocrine part. The absolute phosphorus amounts in the endocrine part are in the region of 1.5  $\gamma$  of phosphorus per  $\mu\text{l}$ . JORPES (loc. cit.) finds in his analyses 2.08 % for the same fractions. Taking into consideration the individual variations in the



Fig. 14. Isolated zymogenous part from the pancreas of a mouse (protocol no. 1 table 34) photographed at 275  $m\mu$ . Enlarged 104 times.

material and the sources of error, however, this difference is not so great. One may in fact say that the ultra-micro method has given satisfactory absolute values also in this case.

*Pancreas of mouse.* — The preparations employed were fixed according to GERSH, and the subsequent treatment was according to chapter 8, II c (p. 64). In one series, only chloroform-extraction was carried out, so that the determinations comprise the sum of acid-soluble phosphorus and residual phosphorus. In table 34 and the two figures 14 and 15 belonging thereto will be found a complete record of some of these experiments. In records no. 3 (figure 15) and 4 is shown the mode of calculation



Fig. 15. Isolated island of Langerhans from the pancreas of a mouse (protocol no. 3, table 34) photographed at 275  $m\mu$ . Enlarged 323 times.

Table 34.

*Analysis protocol from histo-chemical determination of phosphorus in pancreas of mouse (acid-soluble + residual phosphorus), 10  $\mu$  sections.*

	1.	2.	3.	4.
Sample . . . . .	Exocrine	do.	Insular	do.
Plate no. . . . .	7808	7876	7821	7859
Enlargement . . . . .	104 $\times$	104 $\times$	323 $\times$	104 $\times$
Colour-reaction . . . . .	Fiske-Subbarow, ANS no. 22.			
Reaction-volume $\mu$ l . . . . .	1.0	1.0	1.0	1.0
Sector-value . . . . .	384.2	385.0	392.8	390.6
Extinction . . . . .	0.0175	0.0166	0.0079	0.0103
Layer-measurement $\mu$ . . . . .	278	244	252	262
Extinction per cm. . . . .	0.442	0.476	0.219	0.275
Do. corrected for blank value*	0.342	0.391	0.164	0.190
Specific extinction-coefficient $k_c$ . . . . .	135	134	144	143
Phosphorus in $10^{-3}\%$ . . . . .	2.54	2.93	1.14	1.33
Surface of sample on plate, $\text{cm}^2$ . . . . .			35.5	6.94
Do. of exocrine part . . . . .	4.87	7.40	17.5	1.64
Calculated length of edge for preparation considered as a square, $\mu$ . . . . .	212	261	184	254
Sample-volume in $10^{-3}$ $\mu$ l . . . . .			0.340	0.645
Do. of exocrine part . . . . .	0.45	0.68	0.168	0.152
Phosphorus in the exocrine part in $10^{-3}\%$ . . . . .			0.71	0.64
$\gamma$ phosphorus/ $\mu$ l sample . . . . .	5.65	4.27	2.50	1.39

\* The different blank-values in protocol no. 1 and nos. 2—4 are to be explained by the fact that a different binding agent was used for attachment of the section to the collodium membrane.

employed when two kinds of tissue appear in one and the same sample and one knows the phosphorus-content in one of the tissues. For this calculation the phosphorus-content in the exocrine tissue is in this case known (table 35 A).

A summary of all the experiments is given in table 35 A. As may be seen from this table, there is a great difference between the phosphorus-content in the exocrine part and that in the insular part, rather greater than for the corresponding organ from monkfish.

In determinations of corresponding phosphorus-fractions in the whole pancreas of other mammals PLIMMER and KAYA (106)

Table 35.

*Histochemical determination of phosphorus in pancreas of mouse. All the values represent phosphorus in  $\gamma/\mu\text{l}$  organ fixed by freezing-drying.*

*A. Acid-soluble + residual phosphorus. Fiske-Subbarow's method.*

*B. Residual phosphorus. Stannous chloride method.*

	A.		B.	
	Endocrine part	Endocrine part	Endocrine part	Endocrine part
	1.19	4.97	0.58	3.1
	0.54	2.65	0.34	3.0
	0.79	7.13	1.2	3.0
	1.59	4.28	0.67	3.3
	2.30	3.0	1.3	3.4
	1.42	2.74	2.2	3.4
	1.58	3.56	0.5	3.9
	1.24	5.70	2.05	2.9
	1.86	2.50		3.25
	2.50	3.34		3.1
	0.97	5.65		2.6
	1.07	4.24		2.3
	1.39	4.11		4.3
	1.10	4.32		2.9
	0.91	3.20		
	1.49	4.78		
	1.28	3.36		
	1.80	3.65		
	1.06	4.27		
	2.27	2.75		
	0.61	1.94		
	0.41	3.76		
	2.23	8.67		
		2.62		
		7.76		
		4.54		
		4.54		
Mean value .	1.38	4.23	1.1	3.17
$\sigma$ . . . . .	0.52	1.60		0.50
$\epsilon(\text{M})$ . . . . .	0.11	0.31		0.13

found about 0.55 per cent phosphorus (dog) and TEORELL and NORBERG (117) between 0.216 and 0.310 per cent phosphorus (cat). The value of 4.23  $\gamma \text{ P}/\mu\text{l}$  found for the zymogenous part of the pancreas of mouse, that after correction for the shrinking of the preparation and the specific weight of the organ gives

between 0.3 and 0.4 per cent phosphorus, must thus be regarded as very plausible. The acid-soluble phosphorus in the pancreas constitutes according to PLIMMER and KAYA and according to TEORELL and NORBERG about 50 % of the residual phosphorus. Assuming that the distribution is the same in the pancreas of mouse, the nucleic acid phosphorus would be  $0.92 \gamma/\mu\text{l}$  in the insular organ and  $2.82 \gamma/\mu\text{l}$  in the exocrine part. These values are in good agreement with those actually determined by ultra-micro analysis on the same material, as is seen from table 35 B. For the determination of the residual phosphorus, the acid-soluble phosphorus was extracted with 5 % trichlor-acetic acid for 30 minutes after removal of the paraffin with chloroform and alcohol. The sections were then dehydrated with alcohol and moistened with liquid paraffin. The isolation followed according to the description above.

*To sum up*, it may be said that a considerably larger content of acid-soluble phosphorus plus residual phosphorus is demonstrable with the ultra-micro method in the exocrine part of pancreas as compared with the insular organ, both in monkfish and in mouse. Comparison with macro-chemical analysis shows that the exocrine part contains considerably more nucleic acid phosphorus than does the endocrine part, in conformity with the stronger light-absorption at the absorption-maximum of the nucleic acids ( $260 \text{ m}\mu$ ) in the zymogenous part. The difference between the residual phosphorus-content in the exocrine part and that in the endocrine part is demonstrated by ultra-micro determinations.

## CHAPTER 11.

### **The phosphorus-fractions in embryonal and adult hen's liver and in embryonal para-vertebral ganglia.**

*Liver.* — On account of the extraordinarily rapid growth shown by the embryo of the chick between the third and the tenth days of incubation, this material is especially suitable for the investigation of the rôle played by the nucleic acids in the building up of new tissue (CASPERSSON, 37). In investigations on the ultra-violet absorption at  $257 \text{ m}\mu$  in the embryo of the

chick from this period of development, CASPERSSON and THORELL (41) have shown that the liver contains an exceptionally large amount of substances of the nucleic acid absorption type corresponding to a nucleic acid-content of 2—4 % in the cytoplasm. In the liver of the hatched chick, on the other hand, these authors found a considerably lower absorption that could be referred to nucleic acids.

In order to try to verify that this absorption really did derive from nucleic acids, the content of residual phosphorus in liver from an embryo of chick about six days old was determined, as was also the same content from a some weeks old chick. Acid-soluble phosphorus was also determined in parallel experiments. The analyses on embryonal liver were carried out on material that had been fixed according to GERSH (58). The sum of acid-soluble phosphorus and residual phosphorus was determined on sections that had only been extracted with chloroform. For the determination of the residual phosphorus the acid-soluble phosphorus fractions were extracted from a  $10\ \mu$  section with 5 % trichlor-acetic acid, after removing the paraffin with chloroform. After the trichlor-acetic acid treatment the section was dehydrat with alcohol, dried with chloroform and then moistened with paraffin-oil. Subsequent treatment according to chapter 8 IIc. The residual phosphorus in the liver of chick was determined on material fixed with trichlor-acetic acid, while the sum of acid-soluble phosphorus and residual phosphorus was obtained as in the experiments on embryonal liver by analysis of preparation that had been dried by freezing and extracted with chloroform. The results of these experiments are given in table 36.

The content of residual phosphorus found in embryonal liver,  $2.12\ \gamma\text{P}/\mu\text{l}$ , corresponds well with the content of nucleic acid according to the ultra-violet absorption, for when calculated as thymo-nucleic acid the phosphorus value found gives  $3.03\ \text{g}/100\ \text{ml}$ . On conversion to per cent (with the approximative reduction-factor 0.85) one gets the value 2.58 %, which agrees very well with the value calculated by CASPERSSON and THORELL. One is probably justified in considering the phosphorus analyses as a proof that the strong ultra-violet absorption of nucleotide type in embryonal liver is due to a high content of poly-nucleotides.

The nucleic acid-content in the liver of the hen derives chiefly from the cell-nuclei, for CASPERSSON's and THORELL's absorp-



Table 36.

*Some phosphorus fractions in embryonal and adult liver of hen. All the values in  $\gamma$  phosphorus/ $\mu$ l.*

	Embryonal liver		Adult liver	
	Acid-soluble + residual phosphorus	Residual phosphorus	Acid-soluble + residual phosphorus	Residual phosphorus
	4.4	1.02	2.75	0.57
	3.1	1.75	3.52	0.46
	3.1	3.10	2.58	0.47
	4.8	2.10	2.12	1.40
	3.5	3.07	2.94	0.18
	3.27	2.65	2.84	0.41
	2.22	0.96	1.77	0.43
		1.03	2.28	1.16
		2.23	2.40	0.97
		2.47	1.50	0.39
		2.94	1.89	1.36
			1.42	1.02
				1.65
				0.90
Mean values	3.48	2.12	2.32	0.82
$\epsilon$ (M). . . .		0.25	0.18	0.12

tion-data show that the content of cytoplasmic nucleotides is minimal in adult liver. KOSSEL (75) states that the residual phosphorus in the liver of the hen was in two cases 0.083 % and 0.162 %. The value found on the ultra-micro scale, 0.082 g/100 ml, is certainly lower, especially considering that it has to be reduced to give the content as a percentage, but the order of magnitude is nevertheless in good agreement. The content of residual phosphorus in liver from mammals is also in the region of these values. Thus, the residual phosphorus in horse-liver is 0.057 % according to JAVILLIER, CREMIER and HINGLAIS (67), and about 0.08 % in cat-liver (TEORELL and NORBERG, 117).

The acid-soluble phosphorus is clearly about as great in embryonal liver as in adult liver, with the values 1.36 and 1.50  $\gamma/\mu$ l respectively. Converted to per cent we get something over 0.1 %, which agrees with the values given for the liver of mammals, e. g. 0.097—0.118 % for rat's liver (KAY, 70), 0.119—0.131 % for cat-liver (TEORELL and NORBERG, 117) and 0.084 % for horse-liver (JAVILLIER, CREMIER and HINGLAIS, 67).



Fig. 16. Two isolated para-vertebral ganglia from a hen's embryo. Photographed at 275 m $\mu$ . Enlarged 104 times.

*Para-vertebral ganglia.* — In the same embryo of chick as was employed for the above-described experiments with liver determinations were also made on the phosphorus fractions in para-vertebral ganglia, figure 16. The connective tissue surrounding the ganglia was analysed as comparative material. The results of these experiments are found in table 37. From this table it may be seen that acid-soluble phosphorus cannot be demonstrated in the ganglionic tissue. On the other hand, the content of residual phosphorus is extremely high, even higher than in the liver. Also the connective tissue has a remarkably high phosphorus-content. These observations agree on the whole with the strong ultra-violet absorption of nucleic acid type that CASPERSSON and THORELL (*loc. cit.*) have found in the quick-growing embryonal tissue. The high content of residual phosphorus in the ganglionic tissue is also in good agreement with

Table 37.

*The content of acid-soluble phosphorus + residual phosphorus (I) and residual phosphorus (II) in paravertebral ganglia and the residual phosphorus in the paraganglionic connective tissue (III). All the values represent  $\gamma P/\mu l$ .*

	I.	II.	III.
	5.6	3.3	1.7
	3.44	7.4	0.23
	3.9	2.05	2.3
	3.1	7.6	1.4
	3.2	2.0	1.6
	3.1	2.0	2.0
		1.91	1.44
			1.77
Mean . . .	3.72	3.75	1.56

the high content of substances with an ultra-violet absorption of nucleic acid type that is found in nerve-cells according to investigations by LANDSTRÖM, CASPERSSON and WOHLFART (78). Further investigations on the phosphorus fractions in nerve-tissue are, however, called for before one can commit oneself to a more definite opinion.

## CHAPTER 12.

### Determination of phosphorus in single cells (eggs of sea-urchin).

For our present knowledge of the mechanism of development, particular importance attaches to investigations of morphological changes in egg-cells. For biochemical investigations it has been possible to examine single eggs or parts of egg only in special cases (see e. g. 66, 80, 81). As a rule one has had to have recourse to analyses of egg-suspensions with a very large number of eggs. By means of the above-described ultra-micro method, however, that has been tried out on biological material, certain conditions have been created for biochemical studies on the relation of the phosphorus fractions during the development of the cell. In order to test this some preliminary attempts to analyse some phosphorus fractions in single egg-cells have been made. The material comprised unfertilized eggs from the two species of sea-urchin *Psammechinus miliaris* and *Echinocardium cordatum*. Some of the eggs were placed in absolute alcohol and others in Carnoy's fixing fluid. The isolation of the eggs was not carried out until after they had been for several days in the fixing fluid. From a drop of the egg-suspension one egg at a time was transferred to a little drop of liquid paraffin with a dissection needle. After micro-photography, where this was carried out, the egg was transferred to a quartz needle for the phosphorus-determination. The analytic values found are given in table 38.

From alcohol-fixed *Psammechinus* eggs the phosphatide phosphorus is removed as well as 12 % of the acid-soluble fraction according to LINDBERG (82). The ultra-micro determination of the egg thus gives residual phosphorus + 88 % of the acid-soluble phosphorus. On fixing in Carnoy's fluid the phosphatides are removed as well as most of the acid-soluble phosphorus. According

Table 38.

*Some phosphorus fractions in unfertilized eggs of sea-urchin. All the values in  $10^{-3}\gamma$  phosphorus per egg.*

Material	Psammechinus miliaris		Echinocardium cordatum	
Fixation	Absolute alcohol	Carnoy	Absolute alcohol	Carnoy
	0.73	0.65	0.69	0.61
	0.94	0.58	0.93	0.44
	0.68	0.51	0.82	0.56
	0.98	0.64	0.81	0.63
	0.91	0.76	0.61	0.57
	0.67	0.68	0.58	1.09
	0.84	0.33	0.53	0.70
	0.70	0.81	0.40	0.21
	0.76	0.95	0.52	0.54
	0.84	0.95	0.45	0.40
	0.95	0.63	0.58	0.81
	0.85	0.65	0.50	0.61
	0.63	0.30	0.66	0.40
	0.80	0.78	0.53	0.83
	0.63	0.49		
	0.86	0.66		
	0.81	0.52		
	0.79			
	0.87			
	1.13			
	0.81			
	0.98			
	0.85			
	0.92			
Mean value . .	0.83	0.64	0.62	0.60
$\epsilon(M)$ . . . . .	0.025	0.044	0.04	0.12

to LINDBERG (loc. cit.), however, about 5 % of the acid-soluble phosphorus in the eggs is retained (by adsorption?). For the content of total phosphorus and for the different fractions in eggs from sea-urchin the following values are given (ZIELINSKI, 130): total phosphorus 12.80 mg per 100 mg of nitrogen, residual phosphorus 22.5 % and acid-soluble phosphorus about 45 % of the total phosphorus. On the assumption that the conversion-factors given by ÖRSTRÖM and LINDBERG (133) and referring to *Paracentrotus lividus* are also applicable to *Psammechinus* after correction for the size of the eggs (*Paracentrotus* 100  $\mu$ , *Psamme-*

*chinus* 80  $\mu$  in diameter) one gets the following values for the phosphorus-content in a single egg: total phosphorus  $1.7 \cdot 10^{-3} \gamma$ , acid-soluble phosphorus  $0.79 \cdot 10^{-3} \gamma$ , phosphatide phosphorus  $0.38 \cdot 10^{-3} \gamma$  and residual phosphorus  $0.53 \cdot 10^{-3} \gamma$ . The expected amount of phosphorus in an alcohol-fixed egg would then be  $1.2 \cdot 10^{-3} \gamma$ , and in a Carnoy-fixed egg  $0.56 \cdot 10^{-3} \gamma$ . The value for a Carnoy-fixed egg found by ultra-micro determinations,  $0.64 \cdot 10^{-3} \gamma$ , is in splendid agreement herewith. The alcohol-fixed eggs, on the other hand, show a smaller phosphorus-content than one would expect from LINDBERG's analyses, i. e. the acid-soluble phosphorus fraction is too low. A possible explanation of this would be that the long period in the fixing fluid before the analyses (about 5 days) entails that more acid-soluble phosphorus goes into solution in the alcohol than in LINDBERG's experiments. The determinations on *Echinocardium* eggs show that the residual phosphorus (nucleic acid + phospho-protein) is just as great as in the *Psammechinus* egg. On the other hand, no acid-soluble phosphorus is demonstrable, which is probably to be explained in the same way as the low values for acid-soluble phosphorus in the *Psammechinus* eggs.

On the whole one must say that the ultra-micro analyses on phosphorus in single cells have given completely plausible values.

## CHAPTER 13.

### The phosphorus-content in chromosomes from salivary glands of *Chironomus*.

By measuring the ultra-violet absorption in chromosomes from *Chironomus*, CASPERSSON (19) was able to establish as a probability that the nucleic acid-content in the chromosomes is about 10 % or more. In order to ascertain whether the phosphorus-content in the chromosomes was of a corresponding magnitude, phosphorus-determinations were carried out on chromosomes from the salivary gland of *Chironomus Thummi*. The glands were dissected out in Ringer solution and were then left to lie for 1—2 hours in 45 % acetic acid saturated with carmine. The cells were then dissected out whereby the chromosome-ball was often obtained free. Otherwise the cells were crushed under a covering glass. The dissection needles that were employed with the previous prepara-

tion were much too coarse to take up chromosomes with. The best technique proved to be as follows: The selected chromosome or chromosome-group is taken up in a little loop of 10  $\mu$  thick Wollaston wire and quickly transferred to a little drop of paraffinum liquidum. With another 5 or 10  $\mu$  thick Wollaston wire the chromosome is freed from the loop. By means of the fine platinum loop one can now easily transfer the sample to a quartz needle for further treatment. By the above treatment the acid-soluble phosphorus is removed, so that the determination covers residual phosphorus and possibly present phosphatide phosphorus. The analyses are given in table 39.

Table 39.

*The phosphorus-content in chromosomes from the salivary gland of Chironomus Thummi. For the phosphorus-determination the stannous chloride method was employed. The phosphorus-values are given in 10<sup>-3</sup>g. For the re-calculation from one or two to four chromosomes see the text.*

No. of chromosomes	One	Calculated for all four	Two	Calculated for all four	All four from one cell
P . . . . .	0.16	0.52	0.17	0.28	0.25
	0.17	0.55	0.22	0.36	0.23
	0.06	0.19	0.22	0.36	0.25
	0.11	0.35	0.25	0.41	0.48
	0.16	0.52	0.14	0.23	0.48
					0.24
					0.22
					0.34
					0.30
					0.25
					0.25
					0.22
					0.11
Mean value . . .	0.13		0.20		0.28
Mean value for 4 chromosomes (all analyses) . . . . .					0.32
$\epsilon(M)$ . . . . .					0.025 (8 %)

According to BAUER (8), the chromosomes from the salivary gland of *Chironomus Thummi* are in the native state 10–12  $\mu$  in width. The lengths are for chromosomes I–IV in order about 165, 150, 135 and 40  $\mu$ . For the conversion of all values to the same basis it is assumed that "one chromosome" means a 150  $\mu$  long chromosome, and "2 chromosomes" 300  $\mu$ . The sum of the volumes

of the four chromosomes will be  $38.5\text{--}55.4 \cdot 10^{-4} \mu\text{l}$ , and the phosphorus-content herein is, taking the mean of all analyses,  $0.32 \cdot 10^{-3} \gamma$ .

These values give a phosphorus-content in the chromosomes of  $5.8\text{--}8.3 \gamma$  per  $\mu\text{l}$ . Converted to thymo-nucleic acid this gives  $8.3\text{--}11.9 \text{ g/100 ml}$ , which coincides with the value calculated by CASPERSSON.

## Summary.

Theoretical considerations show that with the help of a sufficiently sensitive measuring apparatus it will be possible to extend the range of application of known photometric methods of analysis also to amounts of substance of cyto-chemical magnitudes (the ultra-micro scale). In the foregoing chapters investigations on photometric determinations of phosphorus have been described that confirm this. The essential results of these investigations are the following:

Quantitative photometric determination of phosphorus with the help of one of the coeruleo-molybdate reactions can be carried out on a volume-scale more than two hundred times smaller than that which has hitherto been available for such analyses. With the aid of a micro-photometer, that permits of determinations of light-absorptions to down to 1 % with an error of only a few per cent in the extinction, it is thus possible to determine amounts of phosphorus of cyto-chemical magnitudes. For the special technique that has been worked out for such analyses, FISKE-SUBBAROW's amino-naphthol-sulphonic acid method and DENIGÈS' stannous chloride method are the most suitable. Careful control of the reagents and the conditions for the reactions are necessary. Organic material must first be mineralized before the colour-reaction. On the ultra-micro scale described the mineralization is carried out in the form of ashing, in which connection one must see that there is a sufficient cation-surplus. In the present investigations calcium acetate is used for this purpose. The ashing is shown to give a certain anhydrization of the ortho-phosphate, so that hydrolysis must be performed. On the ultra-micro scale the hydrolysis takes place in a special apparatus, in which the samples can be treated with 1 n hydrochloric acid at 100° for an hour or more. With this technique one can determine phosphorus-amounts of down to  $10^{-4}$  γ, the variation-coefficient going up to



between 20 and 30 % but diminishing with rising amounts of phosphorus.

The isolation and the characterization of biological samples may take place according to several different principles. In certain cases micro-dissection in ultra-violet light with the help of Köhler's microscope is of particular value. In other cases the freezing technique for preparation of sections according to LINDERSTRÖM-LANG and MOGENSEN may be suitable. For the determination of different phosphorus-fractions extraction may be carried out on the isolated sample. The fractioning may also take place by extraction before the isolation, in which connection only the fractions remaining behind in the sample can be determined direct. In the experiments described here the determined fractions have constituted acid-soluble phosphorus, comprising inorganic phosphorus plus a number of phosphoric acid esters, as well as residual phosphorus, the latter comprising nucleic acid phosphorus and possibly existing protein-phosphorus.

In order to test the suitability in different cases of the methods of isolation that were tried out, and the applicability of the method for histo- and cyto-chemical problems, a series of experiments on different material were carried out. On histological material comparative experiments were performed on tissue with strong protein-production and tissue with moderate protein-formation. On cytological material only orienting experiments were performed. The investigations show, in the main, the following:

In rapidly growing tissue, such as the growing zone in onion-roots and embryonal organs, and similarly in protein-producing glands, there was found in all the investigated cases a high content of residual phosphorus as compared with full-grown organs and glands with slight protein-production. As the residual phosphorus as a rule derives only from nucleic acids, this result indicates a high content of nucleic acid. This is in the best agreement with the strong ultra-violet absorption of nucleic acid type that has been observed in corresponding cases by CASPERSSON and others. These coincident observations must be considered to prove that the nucleic acid-content is increased in connection with the formation of protein, which constitutes a very strong support for CASPERSSON's theory (37) that the protein-production takes place with the necessary collaboration of nucleic acids.

The preliminary experiments on cytological material show that the method described can be used for fractioned phosphorus-

analyses both on single cells (egg of sea-urchin) and on the finer cell-details (*Chironomus* chromosomes).

The task that was taken up at the outset of these investigations, namely, by a refinement of the technique to extend the range of applicability of photometric methods also to cyto-chemical problems, has thus been successfully performed as regards analyses of certain phosphorus-fractions.

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ANTITHROMBIN CONTENT  
OF THE BLOOD  
AND ITS RELATION TO HEPARIN



ACTA PHYSIOLOGICA SCANDINAVICA  
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STUDIES ON THE ANTITHROMBIN  
CONTENT OF THE BLOOD AND ITS  
RELATION TO HEPARIN

BY  
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*E. Dahl-Iversen,*

h. a. dec.

*To the Memory of my Father,*

GUSTAV VOLKERT



*Translated from Danish by Hans Andersen, M.D*

## PREFACE

**T**he present work was carried out at the *Biological Institute of the Carlsberg Foundation*, Copenhagen, from January 1941 to March 1942. A brief preliminary report has been published in "*Biochemische Zeitschrift*", and a more detailed account has been given in a paper read before the "Biological Society" of Copenhagen on December 10th, 1941.

I wish to acknowledge my great indebtedness to Dr. ALBERT FISCHER, Chief of the Biological Institute of the Carlsberg Foundation, for the unique working conditions afforded to me and for the kind interest with which Dr. FISCHER has helped me throughout the performance of this work.

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## ORIENTATING REMARKS

Until recently the term "*antithrombin*" has been employed to designate any anticoagulant or thrombin-inactivating substance present in or extractable from the blood or tissues. This term is now known, however, to cover at least two components that are widely different in action and properties.

The first of these, *heparin*, was discovered by HOWEL & HOLT<sup>70</sup> in 1918. It is thermostable, probably a mucoitin-poly-sulphuric acid. Its ant clotting action is immediate, and it can be extracted from various organs, especially from liver and lung. The other component is present in plasma and serum, closely bound to the albumins; it is thermolabile and requires a certain incubation period for exertion of its effect on thrombin. Now the term antithrombin is applied exclusively to the latter component.

Under normal conditions the minimal amounts of heparin present in the plasma are thought to be of no significance to its capacity for inactivation of thrombin. In various pathological conditions such as peptone intoxication and anaphylactic shock, however, it is quite a different matter, as these states of shock, especially in dogs and cats, are associated with a considerable rise in the heparin concentration of the blood. Thus the thrombin-inactivating capacity of the plasma is increased at the same time as the clotting time is prolonged.

Most authors hold that the thrombin-inhibitory effect of heparin and the activity of antithrombin are quite independent of each other.

While we now have a fairly accurate and thorough knowledge of the physicochemical properties of heparin, its effect on the coagulation of the blood and its occurrence in the blood under various pathological conditions, our knowledge of anti-

thrombin is still extremely slight. So far, attempts to isolate it from the blood have been unsuccessful, and the mechanism of its influence on the coagulation of the blood is still obscure; as to variations in the amount of this substance present in the blood in morbid conditions, the reported findings are highly divergent. Our lack of knowledge concerning antithrombin is chiefly due to the fact that until quite recently it has not been possible with any fair degree of accuracy to estimate the amount of this substance in a given specimen of plasma or serum. ASTRUP & DARLING<sup>11</sup> however, in the winter of 1940-41, worked out a method for the quantitative determination of antithrombin with errors less than 5 per cent. All the studies reported in the present work were carried out by the employment of this method for antithrombin determination.

In view of the above-mentioned facts it will be reasonable, in reviewing the literature on antithrombin, to divide these works into two groups. One group comprises the many divergent and conflicting works and theories published prior to the discovery of heparin; the other comprises the modern points of view advanced gradually with our increasing knowledge of this new substance, its occurrence and properties. The introduction of ASTRUP & DARLING's method opened up new possibilities. The purpose of the present studies, therefore, has been by means of this new, precise technique—with some of the older findings for a starting point—to try to give some new contributions to our knowledge of this field in the physiology and pathology of blood coagulation.

## Chapter I.

# SURVEY OF THE MAIN PRINCIPLES IN THE MODERN THEORIES ABOUT COAGULATION OF THE BLOOD

The basis for the modern view of coagulation is the so-called classical coagulation theory. This theory was advanced nearly 40 years ago by MORAWITZ<sup>93</sup>, in 1904, and is founded on investigations carried out by ALEXANDER SCHMIDT<sup>115</sup>, HAMMARSTEN<sup>56, 57</sup>, PEKELHARING<sup>98, 99</sup> and FULD & SPIRO<sup>53, 54</sup>.

Also the studies reported by PANUM<sup>97</sup> on fibrinogen and fibrin as early as 1851 should be mentioned in this connection.

According to MORAWITZ, the process of coagulation is divided into two phases. In the first phase the active coagulant, thrombin, is formed from an inactive precursor through the influence of calcium ions and the tissue factor. In the second phase this thrombin acts on the fibrinogen dissolved in the plasma and transforms it into the insoluble fibrin.

Of the three components required for the first phase, prothrombin—the precursor to thrombin—and the Ca ions are present in solution in the plasma. The third component, the tissue factor, is found in the formed elements of the blood, especially the platelets, and also in all other cells of the organism. In the circulating blood these three components are not able to react with each other and form thrombin, for the tissue factor is separated spatially from the others, being enveloped in the cells. Only when blood is shed the tissue factor is set free, partly from the edges of the wound, partly by disintegration of the platelets, and the coagulation is started by its reaction with the Ca ions and prothrombin.



The second phase of the coagulation, the transformation of fibrinogen into fibrin through the action of thrombin, requires no additional factor.

Since the advancement of MORAWITZ' coagulation theory numerous authors have dealt with this subject and the literature has brought a number of widely divergent or directly conflicting findings and hypotheses. As pointed out by WÖHLISCH<sup>132</sup> (1940) not only had each author his own theory, but in the course of time he has often modified or altered it, so that gradually the coagulation theories outnumbered the investigators working in this field. An additional source of confusion was implied in the circumstance that the terms employed by the various authors did not always cover the same things.

The conflicting experimental results were chiefly due to the fact that the isolated components and systems of components used for investigation were exceedingly impure, so that their properties varied considerably. Gradually, however, as the criteria of the purity of the preparations improved and the preparations became purer, the many different works and hypotheses were taken up for revision, and it was found that only those which were based on the coagulation theory elaborated by MORAWITZ could stand a critical revision. In the past 40 years it has not been necessary to modify this theory or to add any essential feature to it.

While nearly all investigators now subscribe to the scheme set up by MORAWITZ, opinions are still widely divergent as to the physicochemical aspects of the various components involved and their exceedingly complicated reactions to each other.

J. BORDET<sup>19</sup> (1920) is of the opinion that the prothrombin as such does not occur in free form in the circulating blood, but as a precursor ("proserocym"). When blood is shed, contact with rough surfaces and the presence of calcium ions bring about a transformation of this precursor into prothrombin ("serocym") which then reacts with the tissue factor, forming thrombin. According to BORDET, thrombin is a chemical combination of prothrombin with the tissue factor.

Also HOWELL<sup>64, 68, 69, 70</sup> (1911-35) holds that prothrombin is not present in the plasma in the free state but is combined

with a coagulation-inhibiting substance. In his first work he designates this substance as antithrombin, but after he had succeeded in 1918 in isolating a coagulation-inhibiting substance—heparin—from liver extracts, and as this substance subsequently was demonstrated to be present in normal blood although in small amounts, heparin has taken over the rôle of antithrombin. According to HOWELL, heparin acts as an antiprothrombin, but besides this action it also activates a hypothetical substance present in the plasma, transforming it into an inhibitor against thrombin (antithrombin). According to HOWELL, heparin is of importance for the stability of the circulating blood as its combination with prothrombin prevents the transformation of this substance into thrombin, and by its formation of an antithrombin it yields a substance which is able to neutralize the thrombin that may be liberated in the circulating blood under normal circumstances.

When blood is shed heparin is neutralized by the liberated tissue factor, and prothrombin, which now is set free, is transformed into thrombin through the action of the Ca ions.

According to FISCHER<sup>45, 46, 47, 48</sup> (1934–36), thrombin may be regarded as a denaturing enzyme, the action of which may be abolished by heparin, which stabilizes the proteins and protects them against denaturing. Pure fibrinogen is not affected by heparin, however.

FUCHS<sup>52</sup> (1933) combines in part the views advanced by BORDER with those of HOWELL. Thus he thinks that all the factors required for the coagulation are present in the circulating blood. Prothrombin is stabilized, however, by an antithrombin (= heparin) and the tissue factor, which is capable of breaking this combination, is bound and inactivated by the plasma proteins. According to FUCHS, the coagulation is brought about by the circumstance that an additional amount of tissue factor is liberated from the platelets and the edges of the wound. Thus the equilibrium is disturbed, prothrombin is liberated and this substance now combines with the tissue factor in the presence of calcium ions into a chemical compound: thrombin.

Besides these briefly outlined theories, it may merely be

mentioned that a number of authors—*e. g.*, NOLF<sup>95</sup>, MILLS<sup>88</sup>, HEKMA<sup>61</sup>, STUBER<sup>120, 121, 122</sup>—try to explain the mechanism of coagulation as entirely resulting from colloid-chemical changes in fibrinogen, looking upon thrombin merely as a by-product from these processes. But, as mentioned already, this is in conflict with MORAWITZ' theory and not able to stand modern revision and criticism.

For details of the theories briefly outlined above and many other different points of view, the reader is referred to the comprehensive reviews given by WÖHLISCH<sup>130, 132</sup>, HOWELL<sup>69</sup> and OPPENHEIMER<sup>96</sup>.

## Chapter II.

### EARLIER INVESTIGATIONS ON ANTITHROMBIN

The first investigator to discover pathological changes in the coagulation of the blood was SCHMIDT-MÜHLHEIM<sup>116</sup>, who in 1880 found that intravenous injection of WITTE peptone in dogs gave a marked inhibition of coagulation of the blood. In the following years numerous authors took up this problem for further study and, among others, CONTEJEAN<sup>29</sup> succeeded in demonstrating that the failing capacity of the blood for coagulation was due at any rate in part to the circumstance that the injection of peptone resulted in the formation of a new coagulation-inhibiting substance which was assumed to inactivate thrombin. A few years later, in 1901, BORDET & GENGOU<sup>20</sup> were able to demonstrate that immunization of guinea-pigs with fresh serum likewise resulted in the formation of an antibody against thrombin. The authors took this substance to be a species-specific immune antithrombin aimed against the particular thrombin present in the serum employed for the immunization.

ALEXANDER SCHMIDT<sup>115</sup> (1892) was the first to advance the hypothesis that also the normal circulating blood contained a coagulation-inhibiting substance, and although he did not succeed in proving conclusively the existence of this substance, he considered it very important to the stability of the blood.

In 1904 MORAWITZ<sup>91, 92</sup> succeeded in demonstrating in plasma as well as in the serum of normal animals the presence of an inhibitory substance that lowered the action of thrombin markedly. MORAWITZ named this substance antithrombin. According to his statement, this antithrombin was undialysable, and it was destroyed by heating for some time to over 60° and

by the addition of acid or alkali. MORAWITZ assumed that this antithrombin was present in the circulating blood, and played a considerable rôle in the normal coagulation.

In the following years a number of investigators confirmed the observation reported by MORAWITZ. For instance, COLLINGWOOD & McMAHON<sup>27</sup> (1912) showed that an antithrombin was present in human blood, and that the amount of it in plasma and serum was practically the same. These authors regarded it as a proteolytic enzyme aimed against thrombin.

As to the significance of this antithrombin, however, opinions were widely divergent. In contrast to MORAWITZ, some authors thought that the antithrombin was only of slight significance, or none at all. This applies, for instance, to PEKELHARING<sup>100</sup> (1913) who claimed that the coagulation-inhibiting effect of the serum was due to unspecific substances formed after the coagulation; and PICKERING & HEWITT<sup>102, 103</sup> found the assumption of the presence of an antithrombin to be quite unnecessary.

On the other hand, MELLANBY<sup>84</sup> (1909) found that serum contained a great deal of antithrombin, as even small amounts of serum were able to inactivate considerable amounts of thrombin. As the thrombin solution was only a little less active against plasma than against a corresponding solution of fibrinogen, however, MELLANBY concluded that thrombin has a much stronger affinity for fibrinogen than for antithrombin, and that the influence of antithrombin on the normal coagulation, therefore, was only very slight. According to MELLANBY, the effect of antithrombin was not species-specific, since avian thrombin was neutralized by rabbit serum as well as by human hydrocele fluid.

Like MORAWITZ, also NOLF<sup>95</sup> (1913) and HOWELL<sup>84</sup> and collaborators (1911-18) were of the opinion that antithrombin is very important for the preservation of the fluid state of the circulating blood. NOLF attempted to alter the term antithrombin to "antithrombosin", but this suggestion met with no appreciation because his coagulation theory soon proved erroneous. HOWELL, as mentioned already, assigned an important rôle to antithrombin in his new coagulation theory as

well as in the older one, and he and his pupils have reported several extensive studies aimed to elucidate the significance of antithrombin, its properties and its aspects in various pathological conditions.

Thus, DAVIS<sup>31</sup>, a pupil of HOWELL found that intravenous injection of thrombin solutions into various animals produced *no* intravascular coagulation. On the contrary, this treatment resulted in a protracted coagulation time, which he explained as due to an increased antithrombin formation provoked by the injection of thrombin and to the rapid neutralization of the thrombin by this antithrombin. However, he made no attempt to prove this hypothesis. In the opinion of DAVIS, then, the antithrombin played a considerable stabilizing rôle.

In studies on the properties of antithrombin, HOWELL<sup>67</sup> and WEYMOUTH<sup>126</sup>, found that antithrombin was destroyed by heating to over 65–70°, and that even heating to 60° for some length of time would injure the antithrombin considerably. Like COLLINGWOOD & McMAHON<sup>28</sup>, also HOWELL<sup>66</sup> found the activity of antithrombin against thrombin to increase with increasing temperature, the optimum being about 37°. This he considered particularly important to the maintenance of the fluid state of the circulating mammalian blood which in his opinion contained but little antithrombin. According to HOWELL<sup>64</sup> and also WEYMOUTH<sup>126</sup>, the antithrombin was neutralized by tissue extract.

HOWELL<sup>67</sup> emphasized strongly—what had already been realized by MORAWITZ<sup>92</sup> and by MELLANBY<sup>84</sup>—that in order to exert its full activity antithrombin required incubation with the thrombin for a certain length of time. If this condition was not taken into account, the presence of small amounts of antithrombin in the plasma would not be noticed at all.

From these views concerning the nature of the antithrombin HOWELL<sup>67</sup> elaborated his method for determination of antithrombin, which differed to some extent from those employed previously. Its principal features were as follows: The plasma in question was defibrinated by heating to 54° for 5 minutes; then one drop of this plasma was added to a series of tubes containing varying amounts of thrombin; and after 15 minutes'

incubation the coagulation time was measured after the addition of fibrinogen solution. Comparison with a control plasma thus afforded a rough relative estimation of the amount of antithrombin in the plasma. By means of this method, HOWELL<sup>67</sup> found in comparative studies on plasma of various species that the antithrombin content was largest in the rabbit, somewhat smaller in man, and least in the cat. He was also able to demonstrate<sup>65</sup> that lymph obtained from the thoracic duct of the dog contained antithrombin in the same amount as found in the blood plasma.

The statements mentioned agree fairly well with our present view, but they were disputed strongly by various authors. Thus, for instance, PEKELHARING<sup>100</sup> (1913) as well as WEYMOUTH<sup>126</sup> (1913)—in contrast to MORAWITZ<sup>92</sup>—found that it was possible by protracted dialysis to remove the antithrombin from the plasma or, at any rate, attenuate it considerably. PEKELHARING<sup>100</sup> further found that the antithrombin could be extracted with alcohol, whereas MELLANBY<sup>84</sup> (1909) claimed that alcohol destroyed the antithrombin.

As mentioned, one of the principal reasons for the conflicting experimental results was the circumstance that the term antithrombin was applied to all coagulation and thrombin-inhibiting substances present in, or extractable from, tissues as well as the blood, and these substances were all taken to be identical. This conception resulted in several communications showing that antithrombin could be obtained by extraction from various organs, in particular the liver. Especially DOYON and his collaborators<sup>35, 36</sup> (1908-19) investigated this question rather thoroughly and showed that this strong coagulation-inhibiting substance could be obtained from nearly all organs and also from the blood in various ways, particularly by extraction with chloroform, and that this substance was thermostable, being able to withstand boiling even for some length of time. SCHICKELE<sup>117</sup> (1912) likewise obtained a coagulation-inhibiting thermostable substance from the juice pressed out from various organs, especially from the uterine mucosa, but he found this substance to be removed by dialysis. HIRUMA<sup>63</sup> (1923) obtained a similar anticoagulant from the intima, media

and adventitia of the aorta of dogs by simple extraction with physiological salt solution, whereas human aorta yielded no such substance on similar extraction. Also PICKERING & HEWITT<sup>104</sup> (1922), who as mentioned already positively denied the existence of any specific antithrombin, found that coagulation-inhibiting substances could be obtained from various organs by extraction, but they claimed that the same thing happened on extraction of edestin, and that the substances thus obtained, therefore, were simply unspecific split products resulting from hydrolysis of proteins, not physiological cellular products aimed against thrombin.

The view of DOYON, that the anticoagulant obtained by him was identical with the antithrombin occurring in normal blood, was disputed by MINOT<sup>89</sup> (1916) who pointed out that the antithrombin was destroyed by chloroform, and that it was impossible to demonstrate any antithrombin in chloroform extracts of serum and plasma. His statements were later confirmed by P. BORDET<sup>21, 22</sup> (1929).

The appearance of a strongly coagulation-inhibiting substance in peptone blood in dogs and cats, which most authors—according to WÖHLICH<sup>130</sup>, even as late as in 1929—attributed to a simple increase in the inhibiting substance present in normal blood, led to a number of studies and speculations as to the site of the formation of this substance. According to DELEZENNE<sup>32, 33</sup> (1896), CAMUS & CLEY<sup>24</sup> (1910) and NOLF<sup>94</sup> (1910) it was possible to demonstrate a coagulation-inhibiting substance even in the perfusion fluid from an isolated liver perfused with a peptone solution; and the authors took this to indicate that the liver was the site of the formation of this substance. This view was advocated in particular by NOLF<sup>94</sup> who thought he was able to demonstrate that the formation of this substance required a particular cellular activity as it was secreted only when the liver was quite fresh, not if the liver had been lying for half an hour prior to the perfusion. NOLF assumed the capillary endothelium of the liver to be particularly active in this respect.

Also other authors arrived at the conclusion that the liver was at any rate the principal site of the formation of this sub-



stance—*e. g.* POPIELSKY<sup>105</sup> (1913), DOYON<sup>36</sup> (1919) and ARTHUS<sup>3</sup> (1919) who by various ligations excluded the liver from the circulation and then injected peptone intravenously, whereafter they estimated the amount of antithrombin formed. In every instance the antithrombin formation turned out to be lowered considerably, although it had not ceased completely.

By exclusion of other organs or groups of organs from the circulation by means of ligation and estimation of the amount of antithrombin after injection of peptone, POPIELSKY<sup>105</sup> thought he was able to show that antithrombin was formed in other organs, too. In his opinion the antithrombin formation took place in the vascular endothelium of the liver, intestine and extremities. Also ROGER & BINNET<sup>113, 114</sup> (1925) thought that antithrombin could be formed outside the liver—namely, in the lungs.

Employing HOWELL's method for antithrombin determination, DENNY & MINOT<sup>34</sup> (1915) showed that venous blood from the liver, spleen or kidneys contained no more antithrombin than did the blood taken from the jugular vein, but when congestion of these organs was produced, the venous blood from the liver showed an increase in antithrombin. According to these authors phosphorus intoxication produced a fall in antithrombin, indicating that the liver might be the principal site of the antithrombin formation.

PICKERING & HEWITT<sup>104</sup> (1922) tried to disprove the assertion made by DAVIS, that injection of thrombin gave rise to an increased antithrombin formation and that this was the reason why no intravascular coagulation took place. These authors excluded the liver from the circulation by ligation of the vena cava and abdominal aorta, whereafter they injected thrombin intravenously, and yet there was no intravascular coagulation. According to their opinion, this proved that the view of DAVIS was erroneous, and that the fluid state of the blood in the vessels was not due to any antithrombin.

Presumably this absence of intravascular coagulation on injection of thrombin observed by DAVIS & PICKERING was due to the low activity of the thrombin preparations employed for their experiments. Subsequent experiments by other investi-

gators (MELLANBY<sup>85</sup>, ASTRUP & VOLKERT<sup>14</sup>) have shown that intravenous injection of potent thrombin solutions causes immediate death from intravascular coagulation.

BIEDEL & KRAUS<sup>17</sup> (1909) and, a little later, ARTHUS<sup>1, 2, 3</sup> (1910) showed that, besides peptone shock, also anaphylactic shock was associated with inhibition of coagulation—at times, even with total incoagulability—of the blood in the rabbit and especially in the dog, and these authors thought that the two forms of shock involved the same mechanism. PEPPER & KRUMBHAR<sup>101</sup> (1914), ARTHUS<sup>3</sup> (1919) and ZUNZ & LA BARRE<sup>133</sup> (1925) thought they were able to demonstrate that this inhibition of coagulation was due to the formation of an antithrombin. By ligation of the veins of the liver ARTHUS<sup>3</sup> showed that also under such conditions these forms of shock were associated with an increase in the antithrombin content of the blood, though considerably less pronounced. So, in these experiments, too, the liver appeared to be the most important though not the only site of the antithrombin formation.

From the findings mentioned above it thus seems probable that inhibition of the coagulation of the blood associated with peptone shock and perhaps also with anaphylactic shock, especially in the dog, was due to the formation of an inhibitory substance, which was chiefly produced in the liver. Until a few years ago, this substance was considered identical with the antithrombin of normal occurrence, but subsequently it was found to be an entirely different, specific, clotting-inhibiting substance with properties quite different from those of the normal antithrombin. This substance was called heparin—after the organ in which it was first demonstrated, and it was a more thorough investigation of this substance, its properties and occurrence that opened the way for a better understanding of the antithrombin found in normal blood.

In conclusion it will be appropriate briefly to mention the so-called metathrombin, as this has played a great rôle, in particular recently, in the theoretical considerations of antithrombin.

Left standing, a serum soon loses its activity for clotting fibrinogen. MORAWITZ<sup>93</sup> (1905) interpreted this phenomenon as

an inactivation or alteration of the thrombin, and designated this inactive form as metathrombin. As far as is known, this metathrombin is not present in the plasma. Even ALEXANDER SCHMIDT<sup>115</sup> (1892) realized the existence of such a substance in the serum, and he succeeded in showing that it could again be transformed into thrombin by the so-called alkali activation, *i. e.*, by addition of  $n/10$  NaOH and, 15 minutes later, neutralization by addition of  $n/10$  HCl. MORAWITZ<sup>92</sup> showed that also acidification of the serum and subsequent neutralization has the same effect; and later investigations have demonstrated that this reactivation might be brought about in widely different ways.

MORAWITZ thought that the disappearance of thrombin from the serum when left standing was due to polymerization in some way or other. On the other hand, PEKELHARING<sup>100</sup> (1913) claimed that the inactivation was due to the formation of unspecific inhibitory substances that could be inactivated by addition of acids.

MELLANBY<sup>84</sup> (1909) and, later, COLLINGWOOD & MCMAHON<sup>27</sup> (1912) asserted that it was kinase that was set free by the alkali activation, but this view was soon disproved, as the activity of the reactivated serum with regard to fibrinogen was independent of the presence of the calcium ions (GASSER<sup>55</sup> (1917)).

According to studies reported by WEYMOUTH<sup>126</sup> (1913), GASSER<sup>55</sup> (1917) and RICH<sup>111</sup> (1917), the metathrombin is an antithrombin-thrombin combination because, as emphasized by RICH, metathrombin was formed independently of kinase and Ca ions; it could be formed by antithrombin and thrombin, but not in solutions lacking one of these two components. Further, in solutions containing both of these components, the metathrombin formation was associated with a gradual decrease in the amounts of antithrombin and thrombin.

How the linkage of antithrombin-thrombin takes place and how the thrombin-inhibitory effect of serum may be explained will be dealt with more thoroughly below.

### Chapter III.

## RECENT STUDIES ON ANTITHROMBIN

In 1918, HOWELL & HOLT<sup>70</sup> introduced a new component—heparin—into the discussion about the mechanism of the coagulation of the blood. This substance, which had been prepared two years previously by MC LEAN<sup>81</sup>, in HOWELL's laboratory, proved to have an extraordinarily strong coagulation-inhibiting effect. This action HOWELL attributed to two properties: 1) heparin acted as a strong antiprothrombin, and 2) it activated a hypothetical proantithrombin, present in the plasma, into a true antithrombin. According to HOWELL heparin took part in both phases of the coagulation of the blood. It was soon found (1930-40) that heparin could be obtained from most organs (CHARLES & SCOTT<sup>26</sup>, FUCHS<sup>51</sup>, JORPES<sup>75</sup>, KING<sup>76</sup>, MELLANBY<sup>86</sup>, FISCHER<sup>49</sup>, ASTRUP & BEHRNTS JENSEN<sup>7</sup>. According to WILANDER<sup>129</sup> (1939) it is formed by the mast cells, which are particularly numerous in the liver capsule. As mentioned before, heparin was found to be a thermostable substance, probably being a mucoitin-polysulphuric acid, and its inhibitory action was instantaneous.

The discovery of heparin gave HOWELL<sup>68</sup> (1924) the idea that this was the substance given off to the blood stream in peptone shock and caused the incoagulability of the blood in this condition. Indeed, HOWELL was able from peptone blood to prepare a substance which he assumed to be heparin, judging from the procedure employed for its preparation and from the properties it possessed. This has since been confirmed in studies reported by FUCHS<sup>51</sup> (1930), QUICK<sup>107</sup> (1936), WATERS, MARKOWITZ & JAUQUES<sup>125</sup> (1938) and WILANDER<sup>129</sup> (1939) who all found large amounts of a heparin-like substance in peptone

blood. According to WATERS, MARKOWITZ & JAKES the amount of heparin obtainable from the blood of a dog after a peptone shock would be up to one third of the normal heparin content of a dog liver; and according to WILANDER, the amount of heparin in 100 cc. of dog blood after a shock would be between 3 and 6 mg. According to these investigations there appeared to be no doubt that the lack of coagulability of the peptone blood would be due chiefly to an excretion of heparin into the blood stream.

The conditions in anaphylactic shock appear to be analogous. EAGLE, JOHNSTON & RAVDIN<sup>42</sup> (1937) found that inhibition of the coagulation was due to the presence of an inhibitory substance, not to the absence of either thrombin or fibrinogen. WATERS, MARKOWITZ & JAKES<sup>125</sup> (1938) and JAKES & WATERS<sup>73</sup> (1940) could inactivate the shock plasma by adding protamin, which combines with heparin quantitatively, and they also obtained heparin from shock plasma after the method given by CHARLES & SCOTT<sup>26</sup>. They found that both in the rabbit and the dog the blood contained large amounts of heparin after anaphylactic shock, practically the same amount as in peptone blood.

These studies on heparin, its properties and occurrence in various tissues as well as in the blood under conditions of shock threw some new light on many of the older, apparently conflicting investigations. The substance which previous investigators had obtained from the organs and found in the blood in peptone and anaphylactic shock was probably heparin. Also their statements concerning the site of the formation of this substance appeared to be correct, as the liver is particularly rich in heparin, while it is found also in other organs, as emphasized by many of the older authors.

Recent investigations reported by WATERS, MARKOWITZ & JAKES have shown—just like some older studies—that exclusion of the liver from the circulation prior to the shock prevented an excretion of heparin into the blood stream.

In order to obtain evidence for his theory of blood clotting, HOWELL<sup>68</sup> (1924) investigated whether normal blood contains

any measurable amount of heparin, and he succeeded in demonstrating the presence of small amounts of a substance which presumably was heparin. This was confirmed by FUCHS<sup>51</sup> (1930), WILANDER<sup>129</sup> (1939) and CHARLES & SCOTT<sup>26</sup> (1933), but the amount of heparin found was extremely slight. Thus WILANDER gave the heparin content of ox plasma as 1-1.5 mg. per liter, and CHARLES & SCOTT found only 60 units of heparin per liter of ox plasma (1 unit = the amount of heparin required to prevent coagulation of 1 cc. of cat plasma in 24 hours). In serum these authors found no measurable amount of heparin.

We shall not here discuss HOWELL's hypothesis about the significance of heparin to the coagulation of normal blood<sup>68, 70</sup>, but merely establish that the exceedingly small amounts of heparin found are not at any rate sufficient to explain all the thrombin-inactivating property of normal plasma and serum. As stated already by MELLANBY and later confirmed by many other authors, a very considerable amount of thrombin can be inactivated even by small amounts of serum or plasma.

HOWELL<sup>68</sup> (1924) claimed that normal antithrombin and the antithrombin resulting from addition of heparin were two different substances, and that the addition of heparin to plasma thus did not increase the metathrombin formation. Only quite recently, however, ASTRUP & DARLING<sup>9, 11, 12</sup> (1941) have succeeded in proving this conclusively.

According to HOWELL<sup>68, 70</sup> (1924) heparin was not able by itself to inactivate thrombin, as a solution of thrombin and fibrinogen coagulated regardless of the presence or absence of heparin. On the other hand, the addition of heparin to plasma had a most powerful thrombin-inhibiting effect, and from this observation HOWELL concluded that blood must also contain a cofactor (= proantithrombin) necessary for the inhibitory action of heparin. This cofactor was thermolabile, and according to the findings of HOWELL it was closely associated with the plasma proteins.

In contrast hereto, MELLANBY<sup>86</sup> (1935) and FERGUSON<sup>44</sup> (1938) took heparin to be an independent, active thrombin-inhibiting

substance, as, according to their investigations, without the presence of plasma it inhibited the coagulation of a solution of thrombin and fibrinogen.

According to QUICK<sup>106</sup> (1938), this observation was due to the fact that MELLANBY employed for his experiments a solution of fibrinogen obtained by precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , and that this solution contained also the cofactor. But on employment of a fibrinogen solution obtained by precipitation with NaCl, the heparin was found to be ineffective. On the addition of a small amount of plasma + heparin to the thrombin-fibrinogen solution, the result was a strong inhibition of the coagulation. QUICK thus confirmed the view of HOWELL: that plasma contains a cofactor necessary to the action of heparin, and that heparin alone has no effect.

Also FISCHER<sup>47</sup> (1936), JAKES & MUSTARD<sup>72</sup> (1940) and ASTRUP & DARLING<sup>9, 12</sup> (1941) hold the view that the presence of plasma is necessary to the coagulation-inhibiting activity of heparin.

While MELLANBY<sup>86</sup> like HOWELL considered the normal antithrombin and the antithrombin appearing on the addition of heparin as two independent factors, QUICK<sup>107</sup> (1936) advanced another theory. According to him, the substance which, combined with heparin gives a strong thrombin-inhibiting effect, is the normal antithrombin of the blood, which according to QUICK (see below) was taken to be identical with, or at any rate closely associated with the serum albumins.

QUICK demonstrated, as later confirmed by JAKES<sup>72</sup> and by ASTRUP & DARLING<sup>9, 12</sup>, that the cofactor necessary was present also in a solution of serum albumin, and that the combination of heparin + cofactor showed the same thermolability as does the normal antithrombin, being destroyed by heating to 67°.

According to QUICK's theory, the albumins of the blood had a specific affinity for thrombin. Normally, however, this affinity was only slight, not nearly so strong as the affinity of fibrinogen for thrombin, and normally these weak-acting albumins were therefore not able to prevent the coagulation, being unable to

exert their effect until fibrinogen had been transformed into fibrin. When heparin was added to the blood, however, this affinity of albumin for thrombin increased markedly and exceeded the affinity of fibrinogen for thrombin. Now, therefore, thrombin combined first with this activated albumin, by which process it was inactivated, so that it could no longer react with fibrinogen.

This theory, however, could not stand a more thorough investigation. According to ASTRUP & DARLING<sup>9</sup> (1941) it is possible by purification of serum albumin to separate 1) a fraction that has no thrombin-inhibitory action, but can be activated by heparin, and 2) a fraction that neutralizes thrombin, but cannot be activated further by heparin. These authors further found that while thrombin combines quantitatively with antithrombin, the combination of heparin + cofactor as well as the combination between this complex and thrombin appeared to dissociate rather markedly. Finally the authors demonstrated that it is possible in ox plasma to inactivate the cofactor by heating to 56°, whereas the thrombin-inhibiting property of the plasma itself was not impaired to any particular extent in this way.

These investigations have since been continued and extended by ASTRUP & DARLING<sup>12</sup>, who found that the heparin cofactor in bovine as well as human plasma disappeared almost completely within a few minutes when the plasma coagulated, no matter whether this coagulation took place by clotting or was brought about by heating to 54–55° for 5 minutes. The antithrombin of the plasma, on the other hand, was again found unchanged in the serum resulting from the heating of the plasma as well as on normal coagulation.

While thus the cofactor alone was extremely labile, the combination of cofactor + heparin was according to ASTRUP & DARLING<sup>12</sup> (1942) considerably more stable, not being destroyed by heat coagulation at 56° or by spontaneous coagulation. On comparing the thermostability of the combination heparin + cofactor and the fraction of albumins that possess thrombin-inactivating properties but were refractory to heparin, ASTRUP



& DARLING found the conditions here to be almost alike, only that heparin + cofactor perhaps was a little less stable than the other combination, especially at lower temperatures.

As recent investigations by BRINKHOUS and collaborators<sup>23</sup> (1939) and ASTRUP<sup>4</sup> (1939) have further demonstrated that plasma also contains a cofactor indispensable to the antiprothrombin activity of heparin. It thus appears that normal plasma and serum contain one or perhaps two components acting as cofactors for heparin in its prothrombin-inactivating and thrombin-inactivating capacity. Besides these unknown factors there must be a third component, since plasma independently of the addition of heparin is able to inactivate thrombin. The question then arises whether the thrombin-inactivating property of plasma and serum is due to this component alone, or whether perhaps heparin together with the cofactor participates in this activity. So far, all investigators with the exception of QUICK—whose theory, as pointed out before, is erroneous—have assumed antithrombin to be completely independent of heparin.

According to both older and more recent observations (MORAWITZ<sup>92</sup>, MELLANBY<sup>84</sup>, WEYMOUTH<sup>126</sup>, HOWELL<sup>64, 66, 67</sup>, QUICK<sup>109</sup> and ASTRUP & DARLING<sup>9, 11, 12</sup>) this normal antithrombin can be characterized by the following properties:

- 1) It is thermolabile, being destroyed completely by heating to 65°, but being somewhat impaired also at temperatures below this level.
- 2) Its effect on thrombin increases with increasing temperature, the optimum temperature being about 37°.
- 3) For the exertion of its complete activity it requires a certain incubation period with thrombin, at least 15 minutes.
- 4) It is closely associated with the albumins of the blood, and on fractionation it follows this fraction almost exactly.
- 5) Its activity increases proportionally to the amount of plasma or serum, and the combination of antithrombin with thrombin is quantitative.
- 6) Antithrombin is destroyed by chloroform and ether.

Influenced by the discovery of heparin and its significance, the view has been advocated, in particular recently, that this antithrombin does not exist as an independent specific substance directed against thrombin, and that the thrombin-inactivating property of plasma and serum is merely due to the unspecific enzyme-inhibiting effect of the serum proteins, especially the albumins, by simple adsorption.

Considering these questions, the problems concerning the formation of metathrombin in serum and the resulting inactivation of the serum have been of great importance.

Shortly after MORAWITZ' discovery of antithrombin, RETTGER<sup>110</sup> (1909) advanced the view that the capacity of serum for neutralization of its own thrombin content and of additional thrombin was due to the fact that the thrombin combined with the serum proteins. According to RETTGER, this combination was rather loose and could be broken by alkali activation. Although RETTGER looked upon the binding of thrombin as a quite unspecific adsorption, he still assigned a great importance to it *in vivo*, as he was of the opinion that the small amounts of thrombin that were being formed continuously were neutralized in this way. At the same time that RETTGER advanced this view, HEDIN<sup>59, 60</sup> (1909) demonstrated that serum was able to inhibit various enzymes—for instance, the rennet enzyme. This had been demonstrated even previously by LANDSTEINER<sup>80</sup> (1900) in the case of trypsin, but HEDIN showed that this inhibition took place through the same mechanism as the one by which enzymes were inhibited by active carbon, *i. e.*, by simple adsorption. Thus HEDIN found that the enzyme-inhibitory action of serum and carbon to some extent was dependent on the temperature and incubation period, and that the combination of serum and enzyme (though only in the case of rennet enzyme) could be broken by addition of acid, by which the enzyme was again set free. According to HEDIN the combinations of serum and enzyme and carbon and enzyme were at any rate partly dissociated. These findings were shortly afterwards confirmed by ERICHSSON<sup>43</sup> (1911) in studies on the inhibitory action of serum and carbon on invertin. Here the in-

hibition was in both instances in some degree dependent upon the incubation period and temperature. These studies prompted LANDSBERG<sup>79</sup> (1913) to see whether similar conditions held good in the case of the inactivation of thrombin by serum. LANDSBERG examined the inactivating influence of various substances on thrombin—for instance carbon, casein, and the commercially manufactured serum albumin—and he thought he was able to demonstrate that they were all capable of inhibiting thrombin considerably, and that this inhibition, as in HEDIN's investigations, to a certain extent was dependent upon the temperature and incubation period, that it was, at any rate partially, reversible, and that the thrombin could be liberated again by alkali activation. From these findings, therefore, LANDSBERG thought it safe to conclude that the thrombin-inhibiting effect of serum was due to simple adsorption.

These investigations were in conflict, however, with the findings previously reported by MORAWITZ<sup>92</sup> (1904)—that various proteins such as milk, egg albumin, etc., when present in the same concentration as the serum proteins, had no inhibitory influence on thrombin. Also some subsequent studies reported by GASSER<sup>55</sup> (1917) were in conflict with the observations of LANDSBERG. Thus GASSER found that thrombin combines much more strongly with serum than with other colloids as caolin, milk, etc., and that metathrombin was an irreversible combination, whereas the enzyme adsorptions were reversible, at any rate in part. Finally, the alkali activation was found to destroy the antithrombin, whereas, according to LANDSBERG, this process merely liberated the thrombin from the serum proteins.

As mentioned previously, GASSER and two other of HOWELL's pupils, WEYMOUTH<sup>126</sup> (1913) and RICH<sup>111</sup> (1917), looked upon metathrombin as a combination of thrombin and a specific antithrombin.

Recently, however, the views presented by RETTGER and LANDSBERG have been advocated again. Thus, QUICK<sup>109</sup> (1938) found—as was later confirmed by WÖHLISCH<sup>131, 132</sup> (1940), JAKES & MUSTARD<sup>72</sup> (1940) and ASTRUP & DARLING<sup>9, 11, 12</sup> (1941)—that on fractionation of the plasma proteins nearly all the thrombin-inhibiting potency remains in the albumin frac-

tion; and QUICK further demonstrated that heating to 67°, which destroys the antithrombin, is also associated with incipient irreversible changes in the albumin molecules. Also the isolated albumin fraction lost its thrombin-inhibiting effect on being heated to this critical temperature.

QUICK did not think, however, that the thrombin-inhibiting property of the albumins was due to a simple adsorption, but rather was of a specific character, as, like GASSER and MORAWITZ, he found that other proteins (*e. g.*, egg albumin) had no thrombin-inhibiting effect. As pointed out above, QUICK assumed that the protein-inhibiting property associated with the albumins was identical with the cofactor required for the formation of an antithrombin from heparin. His theory was however found not to hold true on more thorough investigation. ASTRUP & DARLING<sup>11</sup> (1942) likewise thought that the thrombin-inactivating properties of the albumins must be of a specific nature; they based their view on the afore-mentioned observation that further purification of the albumin fraction could yield fractions that had no antithrombin effect whatever.

Other authors such as LENGGENHAGER<sup>82</sup> (1940) and, in particular, WÖHLISCH<sup>131, 132</sup> (1940) have again taken up the old theory as advocated by RETTGER and LANDSBERG. Like QUICK, WÖHLISCH demonstrated that practically the entire thrombin-inhibiting effect was found in the albumins of the plasma. On the basis of this and the old findings reported by HEDIN, RETTGER and LANDSBERG, he asserted that the antithrombin effect of plasma and serum was due exclusively to the plasma and serum proteins, while metathrombin merely was to be regarded as the binding of thrombin to these proteins by simple adsorption. This view, however, is contrary to the observations reported by MORAWITZ, GASSER, QUICK and ASTRUP & DARLING.

Furthermore, HEDIN mentions himself that MORGENROTH has shown that the toxin-antitoxin combination likewise can be broken by acid activation so that the toxin again becomes free.

Accordingly, the reactivation of metathrombin by acid and alkali activation cannot be taken to indicate that we are dealing merely with an unspecific adsorption, as specific bindings may also be broken by this treatment. Finally, a rather considerable

increase in the antithrombin content of the blood has been demonstrated by various authors under certain pathological conditions. Under the pathological conditions in question no evidence was found for an increase of the albumin content of the blood. This applies in particular to such pathological conditions as the increase in antithrombin on immunization as reported first by BORDET<sup>20</sup> (1901) and later confirmed by DYCKERHOFF<sup>41</sup> (1939), and in obstructive jaundice as demonstrated by BARLIK<sup>15</sup> (1933) and DYCKERHOFF<sup>39</sup> (1940). If these findings prove correct, therefore, they seem to me to point very strongly against the adsorption theory, and it will be more rational, I think, to assume that the thrombin-inhibiting effect of plasma and serum is due to a specific substance or—as this substance now has been demonstrated by various investigators to be closely associated with the albumins—a specific property of these proteins.

As pointed out previously, however, it is also conceivable that heparin or, rather, heparin + cofactor, play a rôle, too; and even though this be a minor rôle under normal conditions, the possibility is by no means excluded that its importance may alter considerably under pathological conditions. Perhaps it may even be the entire cause of the above-mentioned unexplained increase in antithrombic activity.

The studies to be presented below were therefore undertaken primarily with a view to obtain a more accurate knowledge of the quantitative aspects of antithrombin under normal as well as pathological conditions, and then by further investigation of the results obtained here to investigate the problem of the identity of antithrombin.

## Chapter IV.

# DETERMINATION OF THE ANTITHROMBIN CONTENT OF THE BLOOD

In the present work the antithrombin content of the blood has been determined by employment of a method described by ASTRUP & DARLING<sup>11</sup>. The principle of this method is the addition of various amounts of serum to 1 cc. of a thrombin solution of known strength, incubation of this mixture in a water-bath at 37° for 15 minutes, and measuring of the activity of the mixture by adding a solution of fibrinogen. By comparison with a control solution free from serum it is possible to calculate how much of the thrombin is left, and the amount of antithrombin in the serum is equal to the amount of thrombin that has disappeared. The amount of antithrombin capable of inactivating 1 thrombin unit is used by ASTRUP & DARLING as 1 antithrombin unit, and the amount of antithrombin units in 1 cc. of serum is thus equal to the number of thrombin units inactivated by 1 cc. of the serum.

### Reagents.

1. *Thrombin*.—The thrombin employed for these measurements was a preparation manufactured by "Løvens kemiske Fabrik", Copenhagen, after the method described by ASTRUP & DARLING<sup>8, 10</sup>, the principle of which is: isoelectric precipitation of the diluted plasma by addition of acid to pH 5.3, activation by means of thrombokinase from ox lung and isolation of the resulting thrombin by precipitation with acetone.

According to ASTRUP & DARLING<sup>13</sup>, the potency of this preparation is best determined by comparison with a standard preparation.

The thrombin solution employed was prepared by dissolving 50 mg. of the above-mentioned dry preparation in 15 cc. dilute veronal buffer solution (THORDARSON<sup>123</sup>) with a pH = 7.4. A drop of octyl alcohol is added to this solution in order to prevent foaming, and after standing at room temperature for 1 hour, and filtering, the solution was ready for use. In my studies I have employed 3 different thrombin preparations, and in making up the solutions one of these preparations was found to give a solution containing 40 thrombin units per cc., while another gave 41 thrombin units, and the third 39.6 thrombin units per cc. These standardizations were kindly carried out by Mr. TAGE ASTRUP.

2. *Fibrinogen*.—The fibrinogen preparation here employed was ammonium sulphate-precipitated fibrinogen prepared from oxalated ox plasma according to ASTRUP & DARLING<sup>13</sup>. The fibrinogen was diluted with the above-mentioned veronal buffer solution, and distilled water was added in such an amount that the addition of 1 cc. of fibrinogen solution to 0.1 cc. of control thrombin solution at 37° gave a suitable coagulation time (= about 8–12 seconds). The addition of distilled water to the fibrinogen solution shortened its coagulation time, owing to the reduction in the ionic strength, as, according to ASTRUP<sup>5, 6</sup>, the reactivity of fibrinogen is highly dependent upon the ionic strength of the solution. After this dilution the fibrinogen solution had to be kept on ice throughout the experiment as otherwise it denatures.

3. *Serum*.—The serum was prepared from oxalated plasma by heating in water-bath at 54–56° for 5 minutes, followed by centrifuging.

### Details of the Method.

In a series of test-tubes ( $8.5 \times 1.5$  cm.) 1 cc. of the thrombin solution was pipetted off into each tube and the serum under analysis was added in increasing amounts. The following amounts of serum were used: 0.05, 0.10, 0.15, and 0.20 cc. Then 0.9 per cent NaCl was added to each tube in amounts sufficient to make up a total volume of 1.3 cc. in every tube. After this the mixture was placed in a water-bath at 37° for 15 minutes.

After the incubation the tubes were placed in ice-water. In order to examine how much thrombin had been activated, the activity of the various solutions was then tested against a solution of fibrinogen. These measurements were carried out at  $37^{\circ}$  in a transparent water-bath, and the coagulation times were determined by means of a stop-watch, by recording the appearance of the first floccules of fibrin under gentle shaking of the tubes. For each serum concentration 3 determinations were made. Comparison of the coagulation times measured in this way with the coagulation time for a control solution without serum (1 cc. of the thrombin solution + 0.3 cc. of 0.9 per cent NaCl) afforded a measure for the antithrombin content of the serum employed.

If the clotting time for the control solution is called  $t_0$  and the clotting time observed for one of the serum mixtures is called  $t_m$ , while the amount of thrombin per cc. of the solution employed is expressed by  $a$ , it is evident that, as  $t_0$  corresponds to an amount of  $a$  thrombin units (T.U.),  $t_m$  will correspond to  $a \cdot \frac{t_0}{t_m}$  T.U. Originally the solution contained  $a$  T.U. and  $\left(a - a \frac{t_0}{t_m}\right)$  T.U. had thus been inactivated, corresponding to the amount of antithrombin which had been present in the amount of serum added. So the amount of antithrombin units (A.T.U.) per cc. ( $= x$ ) could be calculated from the equation:

$$x = \frac{a}{b} \left(1 - \frac{t_0}{t_m}\right),$$

$b$  being the amount of serum in the solution employed.

The amount of antithrombin is not determined merely by examination of one serum concentration, however, but by examination of 4 concentrations as mentioned above. From the values obtained in this way a curve was constructed by plotting the added amounts of serum along the axis of abscissas and the values for  $\frac{t_0}{t_m}$  as ordinates. This curve gave a straight line, from which the corrected value for  $\frac{t_0}{t_m}$  could be



read for a suitable amount of serum. By substitution of this reading and the corresponding amount of serum in the above equation the amount of antithrombin per cc. was obtained with a high degree of accuracy.

A numerical example of this is given in Table 1.

From these values  $\frac{t_0}{t_m}$  and the corresponding amounts of serum, a curve was constructed as shown in Fig. 1.

*Table 1. Examples of Values Obtained by Determination of the Antithrombin Content of Serum.*

Incubation			Coagulation time in sec.				$\frac{t_0}{t_m}$
Thrombin ml.	Serum ml.	0.9 per cent NaCl ml.	$t_1$	$t_2$	$t_3$	$t_m$	
1,0	0,00	0,30	10	10	10	10	1,00
1,0	0,20	0,10	43	42	41	42	0,238
1,0	0,15	0,15	21	22	23	22	0,455
1,0	0,10	0,20	16	16	16,5	16,2	0,616
1,0	0,05	0,25	12	12,5	12,5	12,3	0,815

The value  $\frac{t_0}{t_m}$  for 0.2 cc. of serum was derived from the curve as = 0.24. When this value is substituted in the equation, and when the strength of the thrombin solution employed is 41 thrombin units per cc., we have:

$$x = \frac{41}{0.2} (1 - 0.24) = 156 \text{ antithrombin units (A.T.U.) per cc.}$$

### Accuracy and Sources of Error.

Apart from a few particular instances, the experimental animals here employed were ordinary commercial rabbits, especially albino rabbits, weighing between 2 and 3 kg.

The antithrombin determinations were carried out on serum obtained as follows: With a Record syringe containing 0.3 cc. of a 3.5 per cent solution of sodium citrate, 2.7 cc. of blood was taken from an ear vein; and after thorough mixing it was centri-

fused for 10 minutes at a rate of about 3000 revolutions per min., whereafter the plasma was pipetted off. This plasma was then treated by heating as described by ASTRUP & DARLING<sup>11</sup>.

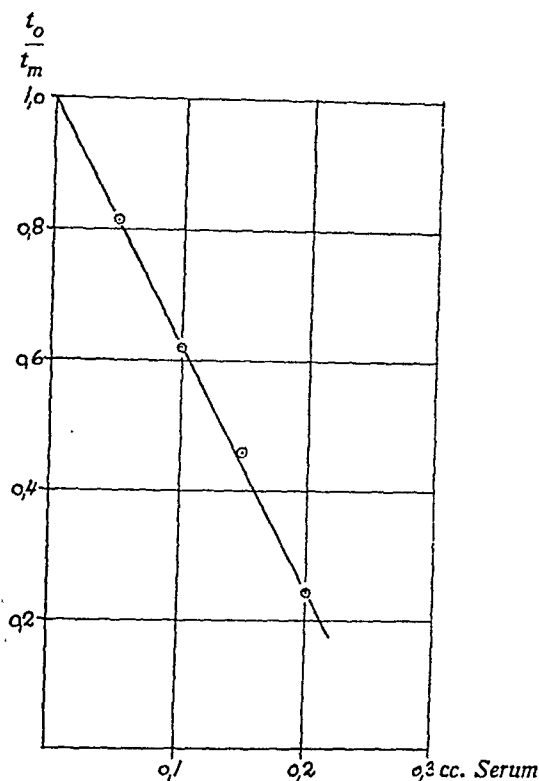


Fig. 1. Curve for determination of the corrected value for  $\frac{t_0}{t_m}$ .

The measurements were carried out on rabbit serum prepared as described above, when no other indications are given.

In some of my experiments, however, it was more serviceable, in order to avoid heating to 54–55°, to be able also to determine the amount of antithrombin in the plasma. For this purpose I employed the same procedure as just described, with the simple modification that here citrated plasma was added to the thrombin solution instead of serum. Owing to the high dilution of the plasma with this technique, the resulting clot was easily broken on shaking the tube after the incubation, and the fluid liberated in this way was added to the fibrinogen in the usual manner. With this procedure, as shown in Table 2, the values obtained

for the amounts of antithrombin were quite in agreement with those obtained with the employment of serum.

*Table 2. Comparison between the Antithrombin Content of Serum and Plasma.*

Rabbit No.	A.T.U. per ml. serum	A.T.U. per ml. plasma
64	155	158
65	157	155
66	156	159
67	157	155
69	154	157
76	156	159
77	153	157
78	157	156
79	155	157

So the results obtained by antithrombin determination on the serum and on the plasma are comparable without correction.

As to this method, the question naturally suggested itself whether eventual changes in pH or in the ionic strength might have some influence on the results obtained.

In my first experiments I used 0.9 per cent NaCl as solvent for thrombin and fibrinogen. When it turned out that I had to work with shock blood the pH of which might deviate from the normal and thus perhaps influence the measuring, I soon went on to employ a buffer solution; and for the sake of safety I subsequently employed this solvent in every instance—not merely when dealing with shock blood. As mentioned already, I have used a veronal buffer solution with  $\text{pH} = 7.4$ , which stabilized the pH of the thrombin and fibrinogen solution at this value, so that possible changes in the pH of the blood had no influence on the results obtained.

The small changes that might occur in the ionic strength of the serum or plasma can hardly have played any rôle due to the great dilution with isotonic solutions. The changes resulting from the addition of distilled water to the fibrinogen for regulation of its reactivity have had an equal influence on the controls

and on the tubes containing plasma or serum, and thus they have had no influence on the result.

The incubation period of 15 minutes at 37° for the mixture of thrombin and serum recommended by ASTRUP & DARLING has likewise been employed in my measurings. The investigations reported by ASTRUP & DARLING<sup>11</sup>, however, were carried out only on ox serum with a relatively slight antithrombin content. So, as my experiments were performed with rabbit serum, one could not be certain that this incubation period would be preferable also in this case. Furthermore, in several experiments I had to work also with sera that contained far greater amounts of antithrombin than did the sera employed by ASTRUP & DARLING.

It was necessary, therefore, to take up the question of the length of the incubation period for further investigation, and for this I employed the technique given by ASTRUP & DARLING<sup>11</sup>.

For each of the sera examined I measured how much thrombin they were able to bind on incubation with the thrombin solution for a varying length of time. The temperature of all the solutions prior to the incubation was room temperature. Immediately after mixing they were placed in a water-bath at 37°, and after the incubation they were placed in ice-water. Then the measurements were carried out as rapidly as possible. From the values here obtained a curve was constructed with the incubation times plotted along the axis of abscissa and the inactivated amounts of thrombin as ordinates (Fig. 2).

Altogether 4 experiments of this kind were carried out on normal rabbit serum, and 11 on sera which, for reasons to be mentioned later, contained a maximum of antithrombin. As seen in Fig. 2, the sera with a normal antithrombin content as well as those with a maximum antithrombin content showed that also with rabbit sera the necessary and adequate incubation period was 15 minutes.

In order to obtain a measure for the accuracy of the antithrombin determinations, 10 independent determinations were made on one specimen of serum, 5 on another specimen. The results are recorded in Table 3.

From this it will be seen that the largest variations occurring in this way amounted to 7 antithrombin units per cc., that is, an error of less than 5 per cent.

Besides this experimental error, resulting from the bleeding, the pipetting and the determination of the coagulation time,

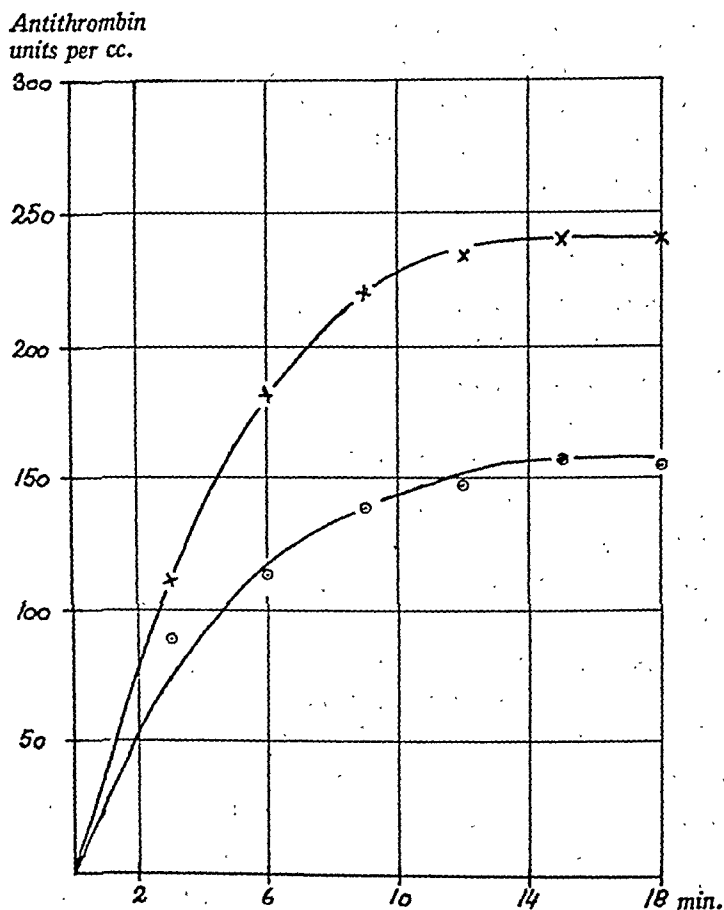


Fig. 2. Curves showing the amount of thrombin inactivated (expressed in antithrombin units per cc.) on incubation with rabbit serum for different lengths of time. The lower curve shows the inactivation of thrombin on incubation with normal serum, the upper with serum containing a maximum of antithrombin.

it was of interest also to learn whether the antithrombin concentration in the individual animal was liable to any particular physiological variation in the course of the day or from day to day. In order to elucidate this question, a total of 34 anti-

*Table 3. Determination of the Accuracy of the Measurements.*

Serum from rabbit No.	Antithrombin measurement No.	Antithrombin units per cc.
12	1	157
12	2	159
12	3	156
12	4	155
12	5	157
12	6	154
12	7	153
12	8	157
12	9	156
12	10	156
9	1	152
9	2	150
9	3	154
9	4	152
9	5	150

*Table 4. Physiological Variations in the Antithrombin Content of the Blood in Rabbit No. 11 through a Period of about 2 Months.*

Date	Antithrombin units per cc.	Date	Antithrombin units per cc.
22/3	158	23/4	158
26/3	152	24/4	154
27/3	152	26/4	154
28/3	164	28/4	156
29/3	156	29/4	156
30/3	152	30/4	154
31/3	154	1/5	156
1/4	154	2/5	154
2/4	164	3/5	156
3/4	156	5/5	155
4/4	160	6/5	156
5/4	162	7/5	156
6/4	152	8/5	156
7/4	156	10/5	162
9/4	152	12/5	158
16/4	156	13/5	155
19/4	154	14/5	154

thrombin determinations were carried out on rabbit No. 11 in the course of about 2 months, during which the specimens of blood were taken at all hours of the day, sometimes after fasting for up to 2 days, sometimes shortly after feeding. The results are given in Table 4.

In this experiment the highest value observed was 164, the lowest 152, that is, the variation was less than 10 per cent.

This variation in one animal corresponded very well to the variation encountered on determination of the antithrombin content of the blood in a number of animals, and I therefore found it unnecessary to repeat the experiment. Thus the antithrombin content of the blood in 80 rabbits was found to be as recorded in Table 5.

From this (Table 5) it will be seen that the variations from one animal to another were slight. Only in one case (No. 7) was the value under 150, and in no instance was it over 160. The average of these determinations is 156 antithrombin units per cc., which therefore may be regarded as the normal value. The amounts of antithrombin recorded in Table 5 are in every instance the value obtained in the first determination performed on the serum of the animals in question. In practice, however, it was found that the value occasionally could be as high as 164 antithrombin units per cc. (see, for instance, Table 4), and the difference between the highest and lowest values here obtained—respectively 146 and 164—is therefore 18 antithrombin units per cc., which gives a variation of about  $\pm 6$  per cent.

As in my experiments it often proved necessary to follow the antithrombin content of the blood from hour to hour over a considerable length of time, and as the complicated method of measuring would make it necessary sometimes to let some of the blood samples stand till the next day, it was essential to investigate the stability of the antithrombin. Examination of the antithrombin content of specimens of plasma after standing for varying lengths of time in the refrigerator gave the results recorded in Table 6.

From these findings the antithrombin content of the plasma—the normal as well as the maximal values—is seen to remain unchanged on standing in the ice-box for a couple of days,

*Table 5. Antithrombin Content of the Blood in 80 Rabbits.*  
 (The numbers lacking here do not refer to rabbits).

Rabbit No.	Antithrombin content of serum	Rabbit No.	Antithrombin content of serum
1	154	45	158
2	150	46	159
3	158	47	159
4	157	48	157
5	160	49	158
6	154	50	157
7	146	51	159
8	156	52	158
9	152	53	155
10	154	54	156
11	158	55	153
12	156	56	155
15	152	57	157
17	154	58	154
18	156	59	155
19	155	60	157
20	155	62	153
21	155	63	156
22	156	64	158
23	157	65	156
24	155	66	159
25	157	67	158
26	155	68	157
27	156	69	156
29	160	70	157
30	157	71	154
31	158	72	154
32	156	73	157
33	156	74	155
34	154	75	156
35	156	76	156
36	155	77	155
37	156	78	157
38	156	79	155
39	154	80	156
40	156	81	155
41	156	82	154
42	157	83	153
43	159	84	153
44	158	85	157



Table 6. *Stability of the Antithrombin Content of the Serum in vitro.*

Animal No.	Condition of animal	A.T.U. in fresh plasma	No. of days in ice-box	A.T.U. in old plasma
1	Normal	152	8	150
11	-	152	6	152
12	-	154	6	154
64	-	155	2	158
65	-	157	2	156
54	Obstructive jaundice	246	2	248
52	-	252	3	250
49	-	246	2	250
51	-	258	3	256
43	Anaphylactic shock	240	2	236
40	(primary rise)	236	11	242
41	-	236	2	246
42	-	240	2	242
61	-	222	4	218
64	Immunization	260	2	252
65	-	254	2	262
66	-	250	2	256
67	-	256	2	256

and even longer storage (up to 11 days) brought no change in this respect. So it is possible to let the plasma samples stand even for several days before the antithrombin determination is carried out.

In conclusion it is to be emphasized that all the antithrombin determinations were carried out on citrate blood in dilution 1:10 and recorded for this. In the studies here reported it is the relative rather than the absolute values which are of importance, and hence I have not found it necessary to carry out any correction of these values.

#### *Recapitulation.*

1. The details of the method described by ASTRUP & DARLING for determination of antithrombin are described, and an account is given of the reagents employed.
2. The necessary and adequate incubation period for the mix-

ture of antithrombin and thrombin is determined for normal as well as for maximal antithrombin values.

3. ASTRUP & DARLING's method is shown to be equally serviceable for plasma and serum.
4. Employment of large amounts of buffer solution eliminates the significance of possible variations in the pH of the blood samples.
5. The accuracy of the method is established.
6. The physiological variations, individual and diurnal, of the antithrombin content of rabbit serum are investigated.
7. The stability of the antithrombin in plasma *in vitro* is examined.

## Chapter V.

# VARIATIONS IN THE ANTITHROMBIN CONTENT OF THE BLOOD DURING IMMUNIZATION AND IN ANAPHYLACTIC SHOCK

### A. Immunization with Proteins.

BORDET & GENGOU<sup>20</sup> observed already in 1901 that during immunization a thrombin-inhibiting substance might occur in the blood. The technique employed by BORDET & GENGOU for demonstration of the increase in the antithrombin content of the blood was as follows: The serum under analysis was heated to 58° for some length of time in order to remove the thrombin. Then it was added to a coagulation system consisting of avian plasma and fresh rabbit serum. Here the avian plasma served as fibrinogen solution, the rabbit serum as thrombin preparation. By comparison of the coagulation times obtained when normal serum and serum from immunized guinea-pigs were added to this system, BORDET & GENGOU demonstrated that the coagulation time with immune serum was much longer than that with normal serum. This they assumed must be due to the fact that the immune serum contained an inhibitory substance directed against the thrombin. On employment of unheated immune serum, however, there was no inhibition of the coagulation, and BORDET & GENGOU took this observation to indicate that the inhibitory substance had no influence on the animal's own thrombin but was directed merely against the thrombin present in the serum or recalcified plasma used for the immunization. BORDET & GENGOU supposed the inhibitory substance to be an antibody produced by the injection of thrombin, and they further thought they were able to demon-

strate that this antibody was species specific and inhibited only the thrombin that originated from the same species as the serum employed for the immunization.

The production of such an immune antithrombin has since been questioned, first by RETTGER<sup>110</sup> (1909), later by MEL-LANBY<sup>85</sup> (1933). Both these authors based their criticism chiefly on their failure through immunization with subcutaneous injection of thrombin to produce any change in the coagulation time of the blood. But neither of them carried out any real antithrombin determination in this connection. Furthermore, as will be pointed out later on, there is no relation between the antithrombin content of the blood and its coagulation time. So the findings reported by these authors cannot be taken as any proof against the observations reported by BORDET & GENGOU.

Nearly 40 years passed before an attempt was made—by DYCKERHOFF & RUHL<sup>41</sup> (1939)—to reproduce the immune antithrombin formation reported by BORDET & GENGOU. In fact, these authors employed the measuring method given by BORDET & GENGOU for the antithrombin determination. They also heated the serum under analysis to 58° for some length of time and added it to a coagulation system containing a fairly uniform amount of fibrinogen and thrombin. As coagulation systems DYCKERHOFF & RUHL employed partly human plasma to which calcium was added at the same time, partly diluted magnesium sulphate ox plasma to which they added a thrombin preparation together with the serum. Like BORDET & GENGOU, they also found it necessary to treat the immune serum by heating, in order to demonstrate the increase in coagulation time, but they interpreted this finding differently, namely as due to the circumstance that the thrombin of the serum was destroyed by the heating and its disturbing influence thus excluded. With this method DYCKERHOFF & RUHL were able to demonstrate that immunization of rabbits is associated with the formation of an antithrombin in the blood and, further, that this antithrombin formation was elicited also by immunizations with proteins containing no thrombin (*e. g.*, casein), and that hence it could not be a specific immune antibody elicited by and

directed against this thrombin. Furthermore, these authors were able to demonstrate that the antithrombin formed under these conditions inhibited thrombin preparations originating from various species equally, ruling out any species specificity.

BORDET & GENGOU's method is very inaccurate, however, even with the modifications given by DYCKERHOFF & RUHL, and allows only of a relatively rough estimation of the changes observed. Thus, for instance, DYCKERHOFF & RUHL found the increase in the coagulation time produced by immune serum in contrast to normal serum to vary from  $-1$  minute to  $+64$  minutes when human plasma was employed in the coagulation system, and from  $+3$  to  $+30$  minutes when bovine magnesium sulphate plasma was employed. Moreover, the results obtained with a serum in one of the coagulation systems would sometimes correspond but poorly to the results obtained with the same serum in the other system.

Probably this uncertainty is due in part to the circumstance that the above-mentioned methods do not include any incubation of the thrombin-antithrombin mixture, and with the short and variable coagulation times the antithrombin could not every time exert its entire activity. This also explains why it was impossible to make any determination on serum that had not been heated beforehand. For when such an unheated serum is employed, its thrombin content causes the coagulation time to be decreased to a few minutes and, as pointed out in Chapter IV, only a fraction of the antithrombin will have time to exert its action within this short period.

From this it is evident that on the basis of the experimental results reported so far, which are conflicting in part, it is difficult with certainty to decide which of them are correct. Furthermore, in none of them has it been possible even approximately to suggest how the demonstrated increases take place, when they appear under the immunization, and what magnitude they reach.

*Writer's Investigations.*

In my experiments on this problem the immunization was carried out with the following protein solutions:

- 1) Bovine BORDET plasma.
- 2) 5 per cent solution of egg albumin.
- 3) 5 per cent solution of edestin.
- 4) 5 per cent solution of casein.
- 5) Human citrate plasma.

These protein solutions were prepared as follows:

The bovine BORDET plasma was prepared by the removal of the prothrombin from bovine oxalate plasma through adsorption on tertiary calcium phosphate. The method employed for this was described by J. BORDET<sup>18</sup>, and it has been described in detail by ASTRUP & DARLING<sup>13</sup>. Because this plasma thus had to be considered free from prothrombin and therefore could not yield thrombin, and also because the Bordet plasma was employed for the preparation of fibrinogen and thus was always available, the greater part of the immunizing experiments were carried out with this plasma.

For the solution of egg albumin a purified powdered preparation (Merck) was used. It was dissolved in slightly alkaline 0.9 per cent NaCl, left standing for two hours and then centrifuged, whereafter it was filtered through a sterile Seitz filter.

The edestin and casein solutions were prepared similarly, only that the former was made up of a purified edestin preparation from the firm »Fränckel & Landau«, the latter of casein »Hammarsten«. The human citrate plasma was prepared by centrifuging blood containing  $\frac{1}{10}$  volume of 3.5 per cent citrate solution.

The immunization was carried out by 5 intravenous injections into the ear veins; each injection dose was 2 cc. The interval between the injections was 48 hours between the two first, 72 hours between the remaining. Altogether 21 immunizing experiments were carried out with this technique on the rabbits, and one experiment on a dog. Of the 21 experiments on rabbits, 13 were performed with injection of bovine BORDET plasma, 1 with egg albumin, 1 with edestin, 3 with casein and 3 with

human plasma. The dog (weight 12.3 kg.) was immunized with bovine BORDET plasma (intravenous injections of 2, 3, 4, 4 and 5 cc.). In addition, two other rabbits (Nos. 43 and 47) were immunized with BORDET ox plasma with the same technique, but they died from protracted shock in connection with the third injection.

By following the antithrombin content of the blood from day to day and by additional measurements in the hours after injection of the antigens, the antithrombin concentration was found to behave as illustrated in Fig. 3, in which the days of the immunization period are plotted along the axis of abscissas while the antithrombin concentrations are recorded as ordinates, expressed in antithrombin units per cc.

This curve is constructed from the antithrombin determinations during immunization with bovine BORDET plasma in rabbit No. 67. The arrows indicate the antigen injections. As will be noticed from the curve, the first two injections produced

*Antithrombin  
units per cc.*

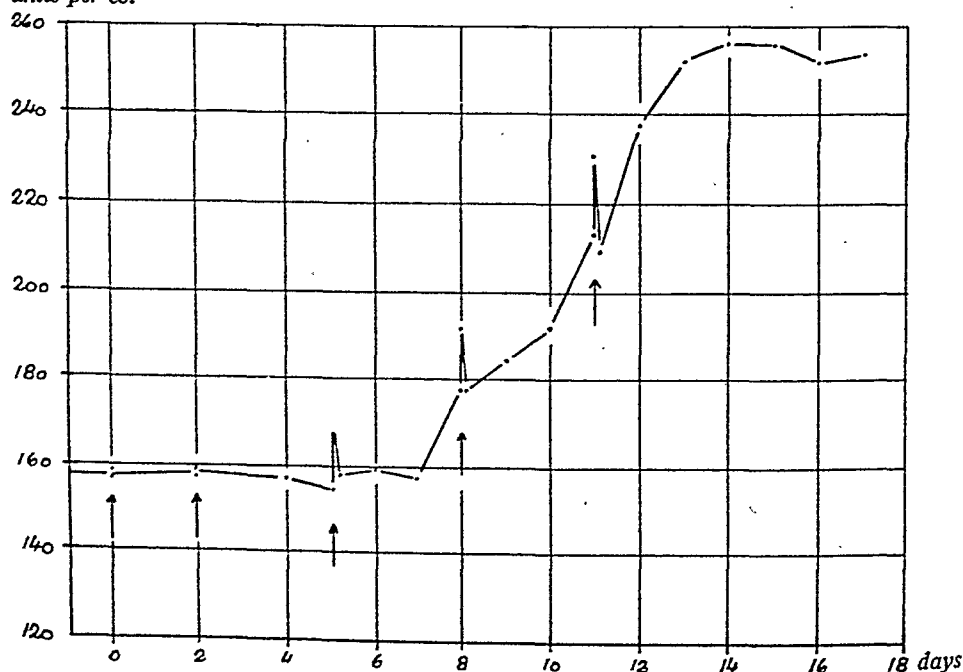


Fig. 3. Antithrombin concentration of the blood during immunization with bovine BORDET plasma. Rabbit No. 67.

→ = intravenous injection of 2 cc. of bovine BORDET plasma.

no change in the antithrombin content. The third injection, on the other hand, gave an abrupt and rapid increase, amounting to about 20 antithrombin units, which was soon followed by a fall to normal level. 24 and 48 hours after the third injection the antithrombin content was still normal, but then it began to rise, at first slowly, later rapidly. Before the fourth injection, that is, 8 days after the commencement of the immunization, the antithrombin content thus had risen to 178, and before the fifth injection—3 days later—it amounted to 213 antithrombin units per cc. After the third injection, moreover, each subsequent injection produced an abrupt though brief reaction appearing on the curve as a peak. After the fifth injection the increase continued for a few days, and then stopped. If the injection of antigen is now discontinued the antithrombin concentration keeps constant at this high level for some days, and then falls off slowly, reaching a normal level in 4–5 weeks.

The course of the process here described has been typical of all the immunizations. Among the unessential variations encountered, it is merely to be mentioned that in a couple of cases the general increase in antithrombin content commenced 24 hours earlier than here described, and that in some cases, the increase has proceeded a little more rapidly, so that the maximum antithrombin concentration was reached prior to the fifth injection, on the 11th day of the experiment.

The more important antithrombin values obtained in these experiments are recorded in Tables 7 and 9.

The slight antithrombin increases amounting to 10–20 antithrombin units, which were brought about by the 3' 4' and 5' injections lasted only a very short time. As early as 1 hour after the injection, the antithrombin content had again fallen off to the value it showed before the injection. Some of the analyses concerning this particular point are recorded in Table 8.

As seen in Tables 7 and 9, practically the same maximum was obtained in 18 of the 21 cases—apart from some minor individual variations, and the increase obtained has thus been about 60–70 per cent.

In 3 of 21 experiments, however, the increase in antithrombin concentration amounted only to about 30 per cent. These 3



Table 7. Antithrombin Content During and After Immunization with Protein Solutions.

Animal No.	Immunized with	Antithrombin content															
		Before immunization	20 min. after 1 <sup>st</sup> inj.	Before 2 <sup>nd</sup> inj.	20 min. after 2 <sup>nd</sup> inj.	Before 3 <sup>rd</sup> inj.	20 min. after 3 <sup>rd</sup> inj.	24 hours after 3 <sup>rd</sup> inj.	Before 4 <sup>th</sup> inj.	20 min. after 4 <sup>th</sup> inj.	24 hours after 4 <sup>th</sup> inj.	Before 5 <sup>th</sup> inj.	20 min. after 5 <sup>th</sup> inj.	24 hours after 5 <sup>th</sup> inj.	48 hours after 5 <sup>th</sup> inj.	5 days after 5 <sup>th</sup> inj.	4 weeks after 5 <sup>th</sup> inj.
10	Bovine Bordet plasma	162	162	150	156	150	174	154	180	188	174	228	240	232	232	244	170
12		158	150	154	154	158	180	164	186	198	180	244	252	240	240	252	168
9	{ 5 per cent egg albumin sol.	152	154	156	158	160	180	160	172	178	213	254	274	256	256		
20	{ 5 per cent edestin sol.	155	154	156	156	163	175	156	197	201	193	242	258	246	266	260	
35	{ 5 per cent casein sol.	156	155	156	155	155	172	156	174	183	177	207	221	205	200		
36		155	156	156	157	157	175	156	174	185	179	204	221	205	205		
37		156	156	157	156	156	180	156	177	181	179	203	217	205	202		
40	Human plasma	156	158	155	156	155	176	157	187	213	193	242	254	250		236	166
41		156	160	158	154	157	174	159	184	205	198	240	260	238		242	162
42		157	158	160	156	158	172	159	186	205	193	230	276	246		234	157
44		158													242		
61		142				140	158		162	174		179	185		174	133	
(Dog)																	
67	Bovine	158	157	158	159	155	168	159	178	192	185	213	231	238	252	252	167
76	Bordet	156													250		
81	plasma	155												246			
82		154												248			
83		153												242			
84		153												246			

*Table 8. Duration of the Small Variations in the Antithrombin Content Produced by Injection of Antigens.*

Rabbit No.	Antithrombin content before 4' injection	Antithrombin content 20 min. after 4' injection	Antithrombin content 1 hour after 4' injection
64	176	188	172
65	169	185	174
66	172	188	176
67	178	192	178
76	204	211	205

*Table 9. Antithrombin Content after the 5' and 7' Antigen Injection.*

Rabbit No.	Antithrombin content before immunization	Antithrombin content 48 hours after 5' injection	Antithrombin content 48 hours after 7' injection
9	152	256	264
64	158	260	266
65	159	254	258
66	159	250	258

animals were the ones that were immunized with casein. So the lesser increase here may be due exclusively to peculiarities of this substance, as it would be difficult to imagine that among 21 animals purchased accidentally I should pick out for my experiment with casein just the three only ones incapable of yielding a great rise in antithrombin concentration.

In the case of the dog the increase in antithrombin was somewhat smaller than the rise presented by the rabbits. Whether this is an accidental observation or whether it holds true of dogs in general, cannot be decided from this single experiment, of course.

That the increase in antithrombin content obtained with the fifth injection is really the maximum that the rabbits are able to produce is evident from the finding that continuation of the injections does not give rise to any additional general increase but merely small and brief antithrombin reactions like those produced by the 3', 4' and 5' injections. Thus, in one instance of immunization with egg albumin, 7 injections were given, and

7 injections were likewise given to 3 rabbits that were immunized with bovine BORDET plasma, and in none of these cases did the last two injections bring any change in the maximum antithrombin concentration obtained with the fifth injection—as shown in Table 9.

In these three cases of immunization with bovine BORDET plasma an attempt was made to continue the immunization beyond the 7 injections, but all 3 animals died in connection with the 8' injection.

In two cases an attempt was made to obtain even a greater increase in antithrombin formation by injection of larger doses of antigen. Thus rabbits Nos. 3 and 4 were given 5 intravenous injections of bovine BORDET plasma within the same period as before but with increasing doses, as follows: 2 cc. at the first injection, and the subsequent injections were increased each time by 2 cc., giving a dose of 10 cc. at the fifth injection. In neither animal did this treatment result in a higher antithrombin concentration than was obtained with the usual dosage of 2 cc. per injection. The large doses proved highly toxic to the animals, however, as they died respectively 12 and 14 days after the commencement of immunization.

Evidently, then, it is possible with 5 injections of 2 cc. in 10–12 days to produce a maximal increase in the antithrombin concentration of the blood in rabbits. So, whenever it has been desirable by immunization to obtain such an increase, the above mentioned technique has been employed in every instance.

In one case (No. 12) an attempt was made a few months after immunization with bovine BORDET plasma to immunize the animal with human plasma. Here, too, the same characteristic antithrombin reaction was observed as during the first immunization.

These experimental results are thus seen to confirm the statements made by BORDET & GENGOV and DYCKERHOFF & RUHL as to the appearance of an increase in the antithrombin concentration during immunization. They further confirm the observations made by DYCKERHOFF & RUHL: that this increase took place regardless of whether or not the protein solutions employed for the immunization contained any thrombin. From my ex-

periments this increase appeared also as a response to immunization with edestin, egg albumin and casein. Finally, my results confirm the observation that the antithrombin formed in this way is not species specific, as in every instance it has been able to inactivate ox thrombin. The increases in antithrombin observed in my experiments have been of a fairly uniform magnitude, however, not varying markedly from one animal to another or from one antigen to another, contrary to the findings reported by DYCKERHOFF and collaborators.

### B. Immunization with Suspensions of Corpuscular Antigens.

Having thus examined the antithrombin content of the blood on immunization with protein solutions, I then tried immunization with bacterial vaccines in order to see whether such treatment gives similar results.

The immunization was carried out on 3 rabbits (Nos. 31, 32 and 12) with typhoid vaccine from the State Serum Institute. This vaccine contained between 1000 and 2000 million bacteria per cc. As in the preceding experiments with immunization, a total of 5 injections were given intravenously, the first two at an interval of 2 days, the following at an interval of 3 days. The dose at the first two injections was 0.25 cc. of vaccine (= 250-500 million bacteria); at the following three injections it was 0.5 cc. (= 500-1000 million bacteria). Prior to the injection each dose of vaccine was diluted with 0.9 per cent NaCl to a volume of 2 cc.

By following the antithrombin content of the blood in these animals, this immunization was found to behave differently from the preceding immunizing experiments. Here the animals showed no general increase in antithrombin concentration, and after 5 injections the antithrombin content was still normal. On the other hand, the last three injections gave an abrupt but brief and slight increase in antithrombin content amounting to about 20 units—just as on immunization with protein solutions (see Fig. 4).

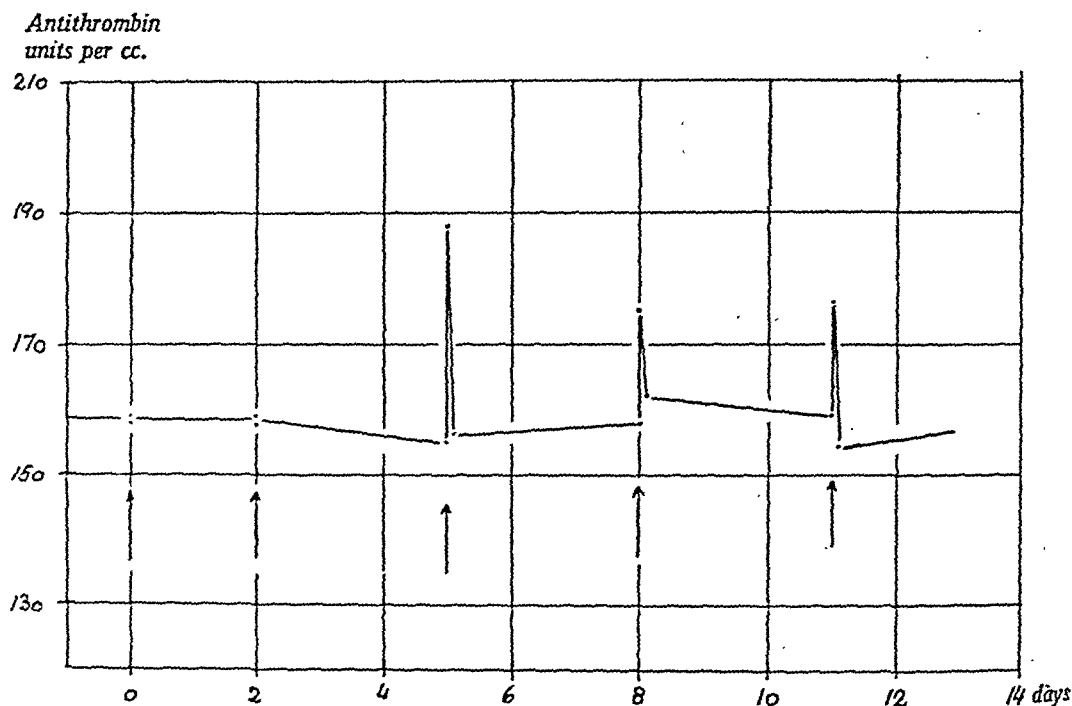


Fig. 4. Antithrombin content of the blood during immunization with typhoid vaccine. Rabbit No. 12.

→ = intravenous injection of the vaccine.

Besides these three cases, in which I performed the immunization myself, I have had occasion to examine the blood of 3 rabbits immunized with pneumococcus vaccine. These 3 animals (Nos. 7788, 7789, 7790) were immunized in the Pneumococcal Department of the State Serum Institute with a polyvalent pneumococcus vaccine of Types 1, 2, 4 and 5, containing about 1000-2000 million bacteria per cc.—like the typhoid vaccine. Of this vaccine 5 intravenous injections were given at intervals of two days, with the same dosage as employed for the typhoid vaccine. Dr. F. KAUFFMANN, chief of the Pneumococcal Department, was kind enough to give me samples of blood from each of these animals taken before immunization and 2 days after. Examination of these blood samples confirmed my previous results as the antithrombin concentration was found to be normal during the immunization (see Table 10).

As the injection of these bacterial vaccines introduced but very small amounts of protein into the blood stream of the animals,

Table 10. Antithrombin Content on Immunization with Suspension of Corpuscular Antigens.

Rabbit No.	Immunized with	Antithrombin content per cc.														
		Before immu- niza- tion	20 min. after 1' inj.	Before 2' inj.	20 min. after 2' inj.	Before 3' inj.	20 min. after 3' inj.	24 hours after 3' inj.	Before 4' inj.	20 min. after 4' inj.	24 hours after 4' inj.	Before 5' inj.	20 min. after 5' inj.	24 hours after 5' inj.	3 days after 5' inj.	4 weeks after 5' inj.
12	Typhoid vaccine	158	159	158	159	155	188	158	175	157	159	176	160	157		
31		158	154	154	158	156	180	158	156	172	157	158	180	157	155	157
32		156	156	160	156	156	178	156	155	177	157	156	177	156	154	159
33	Human erythro- cytes (ghosts)	156	155	156	154	155	178	157	177	157	157	157	177	156	157	159
34		154	156	155	156	156	172	155	156	178	156	156	175	158	156	160
7788	Pneumococ.	158													164	
7789	Vaccine	156													156	
7790		156													162	
68	Yeast.	157	158	158	157	158	183	157	173	159	165	162	154			

(micro-KJELDAHL analysis showed merely a trace of nitrogen in the undiluted vaccine) I tried whether the same features might be obtained by injection of small doses of a protein solution. Thus 2 rabbits were immunized with 5 injections of only 0.05 cc. of bovine BORDET plasma. This treatment gave no reaction in the antithrombin content whatever, although the amount of protein injected here was several times larger than the amount injected during immunization with the vaccine. So the total absence of an antithrombin reaction by immunization with the vaccine appeared not to be due to the small amounts of protein injected.

In order to confirm this finding, in my next experiment I employed for immunization a suspension of the stroma of washed human blood corpuscles. This suspension was prepared in the following way: 18 cc. of blood was withdrawn from a median cubital vein with a Record syringe containing 2 cc. of 3.5 per cent citrate solution. This specimen was centrifuged for 10 minutes at a rate of 3000 revolutions per minute, and the plasma was pipetted off. Then the blood corpuscles were mixed with 10 volumes of distilled water and left standing for 1 hour. The resulting corpuscular stromas (ghosts) were separated by centrifuging for 1 hour (3000 revolutions per min.) and washed by alternating suspension and centrifuging (being washed altogether 6 times—3 times in distilled water, 3 times in 0.9 per cent NaCl). While the stroma suspension in distilled water was very difficult to bring down by centrifuging, so that it was necessary each time to centrifuge for at least 1 hour, the suspension in 0.9 per cent NaCl was easier to deal with and required centrifuging only for about 20 min. After the last centrifuging, the washed corpuscular ghosts were suspended in 4–5 cc. of 0.9 per cent NaCl, and the number of stromas per cc. was estimated by means of a counting chamber.

Immunization with these suspensions was carried out on 2 rabbits (Nos. 33 and 34), as previously, with 5 intravenous injections, each of 2 cc. By suitable dilution of the suspension, each injection now introduced the same number of corpuscular stromas as the number of bacteria given before, but the amount of protein in each injection was here many times greater.

This did not change the experimental results, however, the antithrombin reaction being identical with that which appeared during immunization with bacterial vaccine.

In order to supplement these findings, an attempt was made to immunize two rabbits with a suspension of washed yeast cells from ordinary bakers' yeast. A 10 per cent suspension was employed for this purpose. Injection of this suspension soon proved to be extremely toxic to the rabbits, however, both of them dying after two injections. The experiment was then repeated (rabbit No. 68) but with a 5 per cent suspension of yeast cells that were killed by boiling for two minutes prior to the injections. In this case the animal tolerated the immunization well, showing the characteristic antithrombin variations.

The results of these experiments are recorded in Table 10.

*It appears then that the effect of a suspension of corpuscular elements on the system in which the antithrombin formation takes place differs fundamentally from the effect exerted by the protein solutions.*

### C. Intravenous Injection of High-molecular Substances Without Antigenic Character.

Considering the curve for antithrombin content during immunization (Fig. 3), it will be noticed that, apart from its small peaks, the curve resembles greatly the curve for antibody formation, and it seems obvious, therefore, to assume that the antithrombin formation is associated herewith.

The different curve obtained during immunization with suspensions of corpuscular elements appears to contradict this view, although the difference might be explained—rather artificially, however,—as due, to the fact that precipitin formation on one hand and lysin and agglutinin formation on the other do not proceed entirely through the same mechanism and, therefore, need not have the same effect on the antithrombin formation.

Therefore, in order to throw some additional light on this question, I tried whether it might be possible to produce an increase in the antithrombin content also by intravenous injection of high-molecular substances without antigenic character.



These experiments were carried out on 5 rabbits, employing the following solutions:

- 1) 10 per cent gum arabic solution.
- 2) 5 per cent starch solution.
- 3) 5 per cent gelatine solution.

The preparations used for these solutions were respectively the officinal gum arabic (Danish Pharmacopoeia 1933), soluble starch for analysis, and the ordinary commercial gelatine. All the solutions were sterilized by boiling for  $2 \times 5$  minutes at an interval of 24 hours. At room temperature all three solutions were very viscous, the gelatine even quite solid, requiring heating to  $37^{\circ}$  before their injection.

Gum arabic was injected into two of the 5 rabbits, starch into two and gelatine into one. Each rabbit received 5 injections of 2 cc. and, as in immunization with proteins, the interval between the two first injections was 48 hours, between the others 72 hours.

In these experiments the antithrombin amount was found to increase, and the rise resembled greatly the curve found by immunization with proteins. The general appearance of the curves was the same as well as the small peaks connected with the later injections. On the injection of gum arabic the findings were completely identical with the results from injection of proteins, whereas the general increase in antithrombin concentration on injection of starch and gelatine at first was somewhat slower, and then reached its maximum in about 12–13 days. Similar features were observed in some cases on immunization with protein solutions, so that these minor differences are probably due to accidental individual properties of the rabbits. The results obtained in these experiments are recorded in Table 11.

*It is a particularly striking fact that, in spite of the absence of any antigenic character in the substances here employed, a primary sensitization evidently is required here too, before the appearance of the small peaks. So the two first injections—just as during immunization—showed no effect on the antithrombin content.*

As a supplement to these experiments, I tried whether it might be possible also to produce an antithrombin reaction by the

Table II. *Antithrombin Content on Intravenous Injection of High-molecular Substances without Antigenic Character.*

Rabbit No.	Immunized with	Antithrombin content per cc.														
		Before immunization	20 min. after 1' inj.	Before 2' inj.	20 min. after 2' inj.	Before 3' inj.	20 min. after 3' inj.	24 hours after 3' inj.	Before 4' inj.	20 min. after 4' inj.	24 hours after 4' inj.	Before 5' inj.	20 min. after 5' inj.	24 hours after 5' inj.	48 hours after 5' inj.	4 weeks after 5' inj.
31	{ 5 per cent gelatine sol.	156			154	156	167	164	174	195	189	196	209	230	254	164
32	{ 5 per cent starch sol.	155			153	154	171	158	171	201	189	194	205	222	248	162
60	{ 10 per cent gum. arabic sol.	157	156	156	155	153	169		165	180		205	221		254	152
47		159				157	192	158	189	201		215	234		236	167
48		157				157	189	157	182	205		222	252		234	

employment of a substance of lower molecular size. A rabbit (No. 23) was given 5 injections of 2 cc. of a 5 per cent WITTE peptone solution. This did not result in any antithrombin formation—neither the general rise nor the small brief peaks after the later injections.

Thus the increase in antithrombin content of the blood during immunization has nothing to do with the antibody formation, but is rather to be interpreted as a reaction in response to the injection of high molecular substances.

#### D. Anaphylactic Shock.

As mentioned in Chapter II, it was realized even early that anaphylactic shock is associated with certain changes in the coagulability of the blood, and various investigators thought they were able to demonstrate that under these conditions the thrombin-binding capacity of the blood was increased. The most recent investigations into this question have been published by DYCKERHOFF, MARX & SIEGLER<sup>40</sup>, 1940. Like previous investigators, these authors thought they were able to demonstrate that anaphylactic shock was associated with a rise in the antithrombin content of the blood. In these investigations DYCKERHOFF and collaborators employed the measuring method described by DYCKERHOFF which I have mentioned already. For this reason DYCKERHOFF did not succeed in approaching the problem further than accomplished already by earlier authors, and, like these, DYCKERHOFF had to be content to ascertain that there is an increase in antithrombin in shock, while the details in the mechanism of this increase are still obscure.

The rôle played by heparin in this rise in antithrombin will be discussed in a subsequent chapter.

#### *Writer's Investigations.*

In my studies on this problem the anaphylactic shock was produced in 6 rabbits and 1 dog.

The immunization was carried out as described in the preceding chapter on rabbits by means of 5 intravenous injections of 2 cc. of the antigen employed. In 3 of the rabbits (Nos. 40,

41 and 42) the immunization was performed with human plasma, in the remaining 3 (Nos. 10, 12 and 67) and in the dog with bovine BORDET plasma.

The shock was produced in the rabbits 4 weeks after the treatment by injection into an ear vein of 2 cc. of the antigen solution. In the dog the shock-producing injections were given into the left femoral vein under ether anæsthesia, the dose was 5 cc.; in

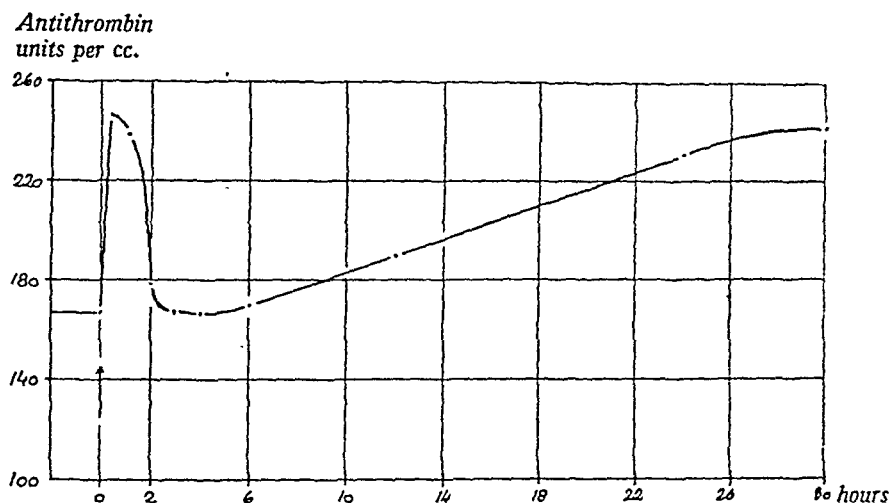


Fig. 5. Antithrombin content of plasma during and after anaphylactic shock produced by sensitization and subsequent reinjection of a protein solution (Rabbit No. 67).

every instance the resulting shock was slight, with merely a few brief convulsions and no loss of consciousness.

Before and, at short intervals, after the shock, samples of blood were taken for antithrombin determination. The values obtained in all these experiments were perfectly concordant. A typical example is seen in Fig. 5.

Immediately after the shock-producing injection the antithrombin concentration increases abruptly by 60–70 per cent, but in the next 2–3 hours this rise is followed by a fall to normal level. In the first hour after the shock the fall is rather slight, but after this it soon becomes abrupt. After having reached a normal value the antithrombin concentration keeps at this level for 5–7 hours, and then it begins again to rise slowly, reaching a maximum about 30 hours after the shock; this maximum is

*Table 12. Antithrombin Content during and after Anaphylactic Shock produced by Sensitization and Subsequent Reinjection of Protein Solutions.*

Animal No.	Antithrombin content per cc.										
	Before shock	20 min. after shock	1 hour after shock	1½ hours after shock	2 hours after shock	3 hours after shock	3½ hours after shock	6 hours after shock	12 hours after shock	24 hours after shock	30 hours after shock
12	156	270	262		238	217		156	178	256	271
40	166	242		197		165		163		232	238
41	162	242			199		160	160	178	244	254
42	157	242		224			157	156	177	244	252
67	167	246	238		178	167		170	190	251	242
61 (Dog)	133	222									

the same as was reached in the primary rise immediately after the injection. Then the antithrombin concentration keeps constant at this level for some days, whereafter it falls slowly, reaching the normal level in 4-6 weeks.

If the injection is repeated in the first days after the shock, it produces merely a slight and brief increase in antithrombin, exactly as observed during the immunization; but here again it is impossible to produce any additional general increase. The maximum reached in the primary as well as in the secondary rise in connection with anaphylactic shock is practically identical with the increase obtained with immunization. A survey of the values obtained in these experiments is given in Table 12.

The duration of the first primary rise in immediate connection with the shock-provoking injection varied somewhat from one animal to another. In 4 of 5 cases a normal value was reached again in 2-3 hours, whereas in one case (No. 12) it took 4 hours.

As the antithrombin reaction on immunization with protein solutions and with suspensions of corpuscular elements differed essentially, it seemed desirable to find out whether this would apply to the anaphylactic shocks also.

In the rabbits which, as described above, were immunized with typhoid vaccine, suspensions of ghosts of red blood cells and suspensions of killed yeast cells, a reinjection of the respec-

tive antigen solutions was therefore performed 4 weeks after the sensitization.

Rabbits Nos. 33 and 34, which have been immunized with suspensions of ghosts of red blood cells were thus given intravenous injections respectively of 4 and 2 cc. of suspension containing about 1000 million particles per cc. In both rabbits this treatment was immediately followed by severe convulsions which in No. 33 led rapidly to loss of consciousness, terminating fatally in 20 minutes. In the case of No. 34, which received half of the dose, the convulsions ceased rather quickly, leaving the animal in a very poor condition, unable to stand on its feet; the rabbit died about 2 hours after the injection.

The blood taken from the jugular vein of No. 33 immediately after death showed an antithrombin content of 232 A. T. U. per cc. In the other rabbit the antithrombin content was found 20 minutes after the injection to be 234 A. T. U. per cc. and 2 hours later it was normal. In these two cases, then, even the fatal shock did not produce any greater antithrombin reaction than the slight ones described previously.

On account of the fatal outcome of the experiment in these two cases, the first of the rabbits that was immunized with typhoid vaccine was first given a preliminary small dose of the vaccine: 1 cc. (= 1000–2000 million bacteria), this did not result in any noticeable shock reaction, and the increase in antithrombin produced by this injection was only slight. In the next two animals, therefore, this dose was doubled. The animals had slight convulsions, and in both of them the antithrombin concentration was increased by about 60–70 per cent. This rise was soon followed by a fall, which was not followed by any secondary rise, the antithrombin content keeping at a normal level during the following days. In the rabbit previously immunized with suspensions of killed yeast cells (No. 68), the shock-provoking injection was a dose of 2 cc. of a 5 per cent solution. In this case too, the antithrombin reaction behaved exactly as recorded for the vaccine injections.

From this it is seen that the antithrombin content here behaved precisely as during the immunization with the suspensions, where the later sensitizing injections produced a brief rise in antithrombin as a reaction against the injection of the antigen,

but the general increase in antithrombin content failed to appear. The results obtained in these experiments are recorded in Table 13.

*Table 13. Antithrombin Content during and after Anaphylactic Shock produced by Sensitization and Subsequent Reinjection with Suspensions of Corpuscular Antigens.*

Rabbit No.	Antithrombin content before shock	Antithrombin content 20 min. after shock	Antithrombin content 24 hours after shock
33	159	232	
34	160	234	
31	157	242	156
32	159	176	156
12	157	248	157
68	154	240	153

As pointed out before in this chapter, intravenous injection of substances without antigenic character gave exactly the same antithrombin reactions as those produced by injection of protein solutions. It thus seemed possible that reinjection of these substances, in spite of their inability to produce shock, would also give variations in the antithrombin content of the blood—possibly the same variations as were typical of the anaphylactic shock, resulting from reinjection of protein solutions.

Therefore, 4 of the rabbits that had received 5 injections of gum arabic, starch or gelatine respectively were 4 weeks later given an intravenous reinjection of 2 cc. of the respective solutions. In spite of the absence of any symptoms of shock, all these animals showed exactly the same antithrombin reaction as encountered in anaphylactic shock, presenting the secondary increase as well as the primary. The results obtained in this experiment are recorded in Table 14.

Two animals sensitized with bovine BORDET plasma (rabbits Nos. 83 and 84) and one sensitized with starch (rabbit No. 60) were about 4 weeks later given an intravenous injection of 2 cc. of a solution of high-molecular substances other than those with which the sensitization had been carried out: No. 60 received an injection of 2 cc. of bovine BORDET plasma; Nos. 83 and 84 were given 2 cc. of a 5 per cent starch solution. This treatment pro-

*Table 14. Antithrombin Content on Reinjection of High-molecular Substances without Antigenic Character. The Injections are made 4 Weeks after the Sensitization.*

Rabbit No.	Sensitized with	Antithrombin content before inj.	Antithrombin content 20 min. after injection	Antithrombin content 5 hours after injection	Antithrombin content 24 hours after injection
31	Gelatine	164	246	168	238
32	Starch	162	234	158	226
60	—	152	234	155	224
47	Gum arabic	167	230	165	234

duced no change in the antithrombin content of their blood. *The antithrombin reaction in anaphylactic shock and on reinjection of starch, gelatine and gum arabic is thus a specific reaction that can be produced only by the very substance with which the sensitization was carried out.* This holds true even when this substance possesses no antigenic character according to the usual conceptions.

This fact, I think, should urge the clinician to be cautious in his employment of unspecific high-molecular substances for intravenous injection, as these experiments show that the organism reacts on their injection, notwithstanding their lack of antigenic character.

According to the findings here reported, the antithrombin reaction is hardly to be looked upon as a part of the shock itself but rather as a concomitant reaction produced by injection of the high-molecular substances. Still, as mentioned already, it is a very peculiar thing that the sensitization appears to play such a great rôle also for substances that are not true antigens.

#### *Recapitulation.*

1. On immunization of 21 rabbits and one dog with intravenous injection of protein solutions, the antithrombin content of the blood is found to increase. This rise is extraordinarily uniform in the various animals and in response to the various antigens, and it is independent of the presence of thrombin in the protein solutions employed. This increase reaches its maximum in 10–12 days. In nearly all the cases this maximal increase amounts to 60–70 per cent of the initial anti-



thrombin concentration; only in 3 rabbits, which were all immunized with a casein solution, and in the dog is the rise somewhat smaller. On discontinuance of the immunization when the maximum is reached, the antithrombin content falls slowly to a normal level in the course of 4–6 weeks.

2. Immunization with suspensions of corpuscular antigens produces no general increase in antithrombin content, but only small and brief antithrombin reactions in response to the later injections, evidently due to sensitization.
3. Intravenous injection of high-molecular substances without antigenic properties—gum arabic, starch and gelatine—gives exactly the same antithrombin reaction as produced by the protein solutions.
4. Examination of the antithrombin concentration during and after anaphylactic shock produced with protein solutions shows first an abrupt primary rise in antithrombin content. This increase is only of brief duration, and after an interval of a few hours in which the antithrombin content lies at a normal level, there appears a new and slow increase which reaches a maximum about 30 hours after the shock. The primary rise as well as the secondary amount to about 60–70 per cent of the initial value.
5. In anaphylactic shock produced by suspensions of corpuscular antigens only the primary rise is produced.
6. Reinjection of high-molecular substances without antigenic properties—gum arabic, starch and gelatine—gives exactly the same increases in antithrombin content as observed in anaphylactic shock produced by protein solution.

## Chapter VI.

# ANTITHROMBIN CONTENT OF THE BLOOD IN OBSTRUCTIVE JAUNDICE AND EXPERIMENTAL INJURY TO THE LIVER

### A. Obstructive Jaundice.

The observation that jaundice is often associated with hæmorrhagic diathesis is of no new date, but until recently very little has been known about the cause of this. In discussions about the relation of these phenomena, however, it was asserted early by a few authors that under such conditions the blood contained an increased amount of thrombin-inhibitory substances and that these caused the decrease in the coagulability of the blood. Thus, for instance, WHIPPLE<sup>127</sup> (1913) thought he had been able in various cases of jaundice to demonstrate the presence of such substances in the blood. This was confirmed a few years later by MINOT & DENNY<sup>90</sup> (1916) who, in a single case of human obstructive jaundice, likewise found an increased amount of antithrombin in the blood. Also HARTMANN<sup>58</sup> (1927) thought he was able in patients with liver lesions to demonstrate an increase in the thrombin-inhibitory substances of the blood. Neither WHIPPLE nor HARTMANN made any distinction between the various forms of jaundice, and looked upon the injury to the liver as the cause of the rise in antithrombin concentration.

BARLIK<sup>15, 16</sup> also subscribed to this view, and found it supported in experimental studies on rabbits in which he produced acute obstructive jaundice by ligation of the common bile duct. The animals were bled 5 days after the operation, and the blood was analyzed. As a measure for the antithrombin con-

centration, BARLIK used the increase in coagulation time produced by addition of the pathological plasma to a mixture of normal plasma and fresh serum. Naturally this method for antithrombin determination was a very rough estimation, and hence the experimental results obtained by BARLIK had to be highly variable as is also evident from his records. Notwithstanding this inaccuracy, BARLIK still thought he was able to demonstrate that the antithrombin content of the blood in the animals was increased considerably after the operation.

All BARLIK's analyses were carried out on blood taken only 5 days after the operation, and he reported no measuring of the antithrombin concentration before or after this time.

The discovery of vitamin K by DAM<sup>30</sup> and the resulting studies on the lack of prothrombin under various pathological conditions diverted for a while the attention from the inhibitory substances.

In 1940 the problems were again taken up by DYCKERHOFF & MARX<sup>39</sup>, whose studies were carried out on rabbits after ligation of the common bile duct. The measuring method employed in these studies was the same as the one used previously by DYCKERHOFF; it has been described already in detail in Chapter V.

In these experimental studies DYCKERHOFF & MARX succeeded in demonstrating that obstructive jaundice is associated with a rise in the antithrombin content of the blood. Also in these studies, however, the inaccuracy of the measuring method has asserted itself, making the results highly variable. It was impossible, therefore, from their results to see whether there was any connection between the duration of the jaundice produced and the rise in antithrombin concentration. In one case, for instance, the authors found a rise in antithrombin content as early as two days after the ligation of the common bile duct as manifest in a prolongation of the coagulation time amounting to 10 minutes, while in another case this result appeared only after 17 days. According to these findings, the time for the appearance of the rise in antithrombin concentration as well as the degree of this rise must be subject to extraordinary individual variations, but their experimental records convey no definite

impression of these conditions, as regular repeated determinations after the operation appear not to have been performed. DYCKERHOFF & MARX had the impression themselves that the rise in antithrombin took place soon after the onset of jaundice—as was evident from their experiments in producing an increase in antithrombin by intravenous injection of bile, since here they measured the antithrombin content of the blood a few minutes after the injection.

WHIPPLE, HARTMANN, BARLIK and DYCKERHOFF & MARX ascribe to these inhibitory substances a considerable rôle in the appearance of hæmorrhagic diathesis in liver lesions.

As is evident from the above, the problems concerning the occurrence of a rise in antithrombin concentration are still far from settled, and thus require additional study.

#### *Writer's Investigations.*

In my studies the experimental obstructive jaundice was produced in rabbits by ligation of the common bile duct under ether anaesthesia by means of a double silk ligature about 1 cm. from the duodenum. The operation was performed with a vertical incision through the skin and subcutis from the right costal margin, for about 5–6 cm. distally, a little medially to the papillary line. After division of the fascia, blunt dissection through the musculature, following the direction of the fibres; then the peritoneum was opened. The duodenum was now found in the depth of the upper angle of the wound, and ligation of the common bile duct was easy; care had to be taken, of course, not to injure the hepatic artery, which at this very place turns behind and under the common bile duct. After ligation, the abdominal wall was closed in layers.

On an average, the rabbits stood the operation well and the majority of them survived the operation for 2–4 weeks.

This ligation of the common bile duct was performed on a total of 8 rabbits. The antithrombin concentration was determined on all the rabbits before the operation and at intervals of 2 days after the operation till the animals died.

In this way, the antithrombin content was found to keep

constant for 4–5 days after the operation, although their blood showed a strong icterus as early as 24 hours after the operation. After the 4'–5' day the antithrombin content commenced to rise, at first slowly, but gradually with increasing rapidity, reaching a maximum in 12–14 days after the operation. Then it kept constant at this level until the death of the animal, no matter whether the animal lived 2–3 weeks longer.

The antithrombin reaction was practically the same in all the animals, corresponding to the course outlined above. A typical instance is shown in Fig. 6.

A survey of all the experiments is given in Table 15.

As shown in Table 15, the maximum antithrombin concentration obtained was subject to some individual variation, but still surprisingly uniform, showing in every instance an increase of 60–70 per cent. The course of the rise as well as the maximum obtained bore a striking resemblance to the findings on immunization. In a subsequent chapter these problems will be dealt with more thoroughly, and the possible cause of the antithrombin reaction will be discussed.

Besides these experiments I have had occasion to examine the antithrombin reaction in an additional case that will be mentioned summarily because of the special conditions connected with it.

This was a rabbit with experimental obstructive jaundice from ligation of the common bile duct. The operation on this animal was performed by Dr. GADE in the Pathological Institute of the Municipal Hospital, Copenhagen. Dr. GADE was kind enough after the operation to let me take some samples of blood for antithrombin determination.

As in my own case, the operation was followed by a rise in antithrombin content, but only till the 12' day when it had reached the value of 216 antithrombin units per cc. The later determinations showed steadily decreasing values till the normal level was reached. At the same time, the icterus of the animal subsided as was plainly manifest in the plasma colour. For this reason, and because the animal seemed to be perfectly well, it was killed about 6 weeks after the operation. On autopsy no trace of the ligature could be found, and the passage through

Table 15. *Antithrombin Content in Experimental Obstructive Jaundice.*

Rabbit No.	Antithrombin content per cc.															
	Before operation	2 days after opr.	4 days after opr.	6 days after opr.	8 days after opr.	10 days after opr.	12 days after opr.	14 days after opr.	16 days after opr.	18 days after opr.	20 days after opr.	22 days after opr.	24 days after opr.	26 days after opr.	28 days after opr.	30 days after opr.
1	154	150	158	166	175	187										
17	154	156	160	168	180	197	217	266	272							
49	158	154	161	168	169	187	217	234	242	242	254	254	250	247	248	246
50	157	154	165	168	175	191	207	224	248							
51	159	157	161	166	182	203	215	228	238	258	255	256	248	251	254	
52	158	154	164	169	175	188	198	200	242	262	265	264	257	255	260	250
53	155	157														
54	156	157	166	170	181	195	207	220	243	246	246					

the common bile duct was perfectly free. Somehow the ligature must have become undone and displaced. This instance shows, then, that the rise in antithrombin resulting from occlusion of

*Antithrombin  
units per cc.*

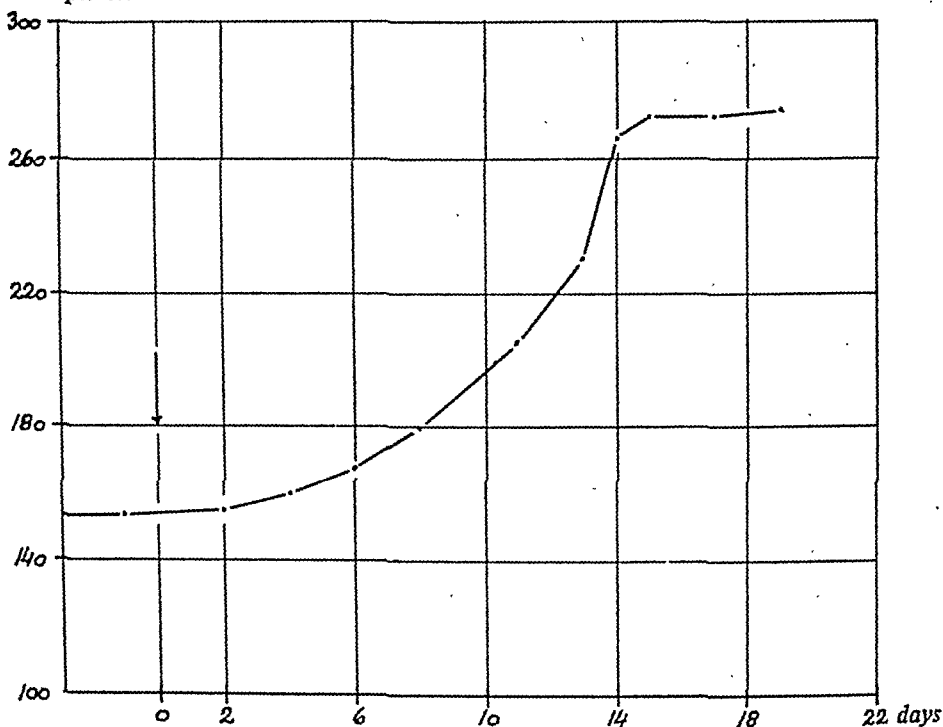


Fig. 6. Antithrombin concentration of the blood in experimental obstructive jaundice.

→ = ligation of the common bile duct.

the bile duct may subside completely when the passage of the duct is again free.

In all my experiments the antithrombin reaction was found not to commence till several days after the operation, and even then it progressed but slowly in the following days.

According to BARLIK's investigations, however, there was a distinct increase in antithrombin already 5 days after occlusion of the common bile duct; and in some of their experiments DYCKERHOFF & MARX found a marked rise in antithrombin even as early as 1-2 days after the operation. In view of the findings presented here, the observations reported by these

authors have to be considered erroneous, presumably because of the poor measuring methods available to them.

Nor have my experiments been able to confirm the wide individual variations found by DYCKERHOFF & MARX in their animals as to the time of the appearance of the rise in antithrombin as well as the magnitude of this rise, for in all my animals I found the rise to proceed after the same rules and the maxima obtained to be practically alike.

### B. Experimental Injury to the Liver.

As mentioned before, WHIPPLE, HARTMANN and BARLIK considered the injury to the liver to be the cause of the increase in antithrombin concentration appearing in obstructive jaundice. Thus, HARTMANN thought the rise in antithrombin was brought about by the circumstance that the liver, which normally should continually give off small amounts of antithrombin to the blood stream, was unable to retain the antithrombin when it became damaged, and that the blood thus was flooded with antithrombin from the liver. According to BARLIK, too, the antithrombin content of the blood is regulated by the liver, and the rise in antithrombin in obstructive jaundice is due to injury to the liver cells, making them incapable of this function.

This conception of the conditions was based entirely on speculative considerations.

Also DYCKERHOFF & MARX seem to have attached considerable importance to the injury done to the liver parenchyma, but without attempting to explain the mechanism of this process.

On the other hand, several authors have reported a number of observations that seemed to go against this view. As early as 1912, for instance, WHIPPLE<sup>128</sup> found no increase in antithrombin concentration in a severe case of cirrhosis of the liver; nor was he able to produce such an increase by chloroform poisoning in dogs.

Also DENNY & MINOT<sup>34</sup> (1915) and MINOT<sup>89</sup> (1916) tried to produce injury to the liver in dogs by means of phosphorus and chloroform, and they succeeded in demonstrating a fall



in antithrombin under these conditions. However, examination of the blood from patients with severe parenchymatous lesions of the liver revealed no change in the antithrombin concentration, DENNY & MINOT tried to explain this discrepancy as owing to the circumstance that the injury to the liver would have to be extraordinarily severe in order to give a fall in antithrombin, as it would require only a relatively small part of the liver to maintain this function of the blood.

This argument seems rather inadequate, however, as it would hold true also when the damage to the liver was produced by obstructive jaundice.

SMITH, WARNER & BRINKHOUS<sup>118</sup> (1937) were unable by damaging the liver in dogs with chloroform to demonstrate any change worth mentioning in the antithrombin content of the blood. Obviously, then, the quantitative aspects of the antithrombin content of the blood in injury to the liver remain unsettled, and opinions on this question differ.

In order to investigate this question, like DENNY & MINOT, I have carried out some experiments with injury to the liver in rabbits, for which I used carbon tetrachloride and chloroform.

#### *Writer's Investigations.*

The poisoning with chloroform was carried out on 6 rabbits. One of them was given a subcutaneous injection of 5 cc. of pure chloroform, which resulted in the death of the animal within 24 hours. Two rabbits received 3 subcutaneous injections of 1 cc. of pure chloroform at intervals of 3 days. Also this dose appeared to be too large, as one rabbit died six days after the last injection; the other survived the poisoning. The remaining three rabbits were given 2 subcutaneous injections of 1 cc. of the same chloroform preparation, 3 days apart. Of these 3 animals 2 survived the poisoning.

Poisoning with carbon tetrachloride was carried out on 1 rabbit with one injection of 10 cc. subcutaneously.

Soon after the administration of these poisons, all the rabbits showed pronounced general symptoms with weakness and

loss of appetite. As mentioned, 3 of the animals died, whereas the remaining 4 recuperated rapidly after some days of illness. Besides the general symptoms, the animals presented superficial areas of necrosis at the sites of the injections, and in the rabbit which was given 10 cc. of carbon tetrachloride (subcutaneously in the right thigh) the entire extremity became paralyzed permanently.

On following the antithrombin content of the blood from day to day, like DENNY & MINOT, I also found a fall in the antithrombin concentration. In chloroform poisoning the antithrombin values were found to be somewhat lower as early as 24 hours after the first injection, and the fall continued during the next two days. Then 1 cc. of chloroform was again given subcutaneously, and this produced an additional fall in antithrombin, though somewhat smaller than the first, and this effect ceased in about 48 hours. The third injection caused again a decrease in antithrombin but only relatively slight and brief as after the second injection.

When the injections were discontinued and the fall produced by the last injection had ceased, the antithrombin concentration commenced again to rise rapidly and reached the normal level in about 4 days. The rise did not stop here, however, but continued to some higher level, and then it began to fall off slowly, reaching the normal level once more after some time. In several cases this secondary rise was rather considerable.

In all the rabbits the antithrombin reactions were identical; a typical instance is presented in Fig. 7.

The rabbit given a subcutaneous injection of 10 cc. of carbon tetrachloride showed changes in antithrombin content which greatly resembled the ones described above, only that the fall here was smaller and more protracted; and in this animal too, there was a secondary rise above the normal level.

The results of these experiments are recorded in Table 16.

Microscopic examination of the liver in the rabbits that died after the injection showed that the poisoning was associated with serious degenerative changes in the liver cells throughout the whole organ. On comparison of such a specimen with the liver from a rabbit, 18 days after ligation of the common bile

Table 16. *Antithrombin Content in Experimental Injury to the Liver.*

Animal No.	Antithr. content before intoxication	Intoxication produced by	Antithrombin content				
			1-2 days after last inj.	4 days after last inj.	6 days after last inj.	14 days after last inj.	21 days after last inj.
19	155	3 inj. of 1 cc. of chloroform	116	147	194		
77	153	-	119	158	187	202	190
22	156	2 inj. of 1 cc. of chloroform	121				
78	157	-	130	157	191	206	210
79	155	-	113	142	188	200	198
18	156	1 inj. of 10 cc. of carbon tetrachloride	154	148	140	141	167

duct, extensive injury to the liver tissue is seen in both instances (Figs. 8 and 9).

As the injury to the liver in chloroform poisoning thus gives

*Antithrombin*  
units per cc.

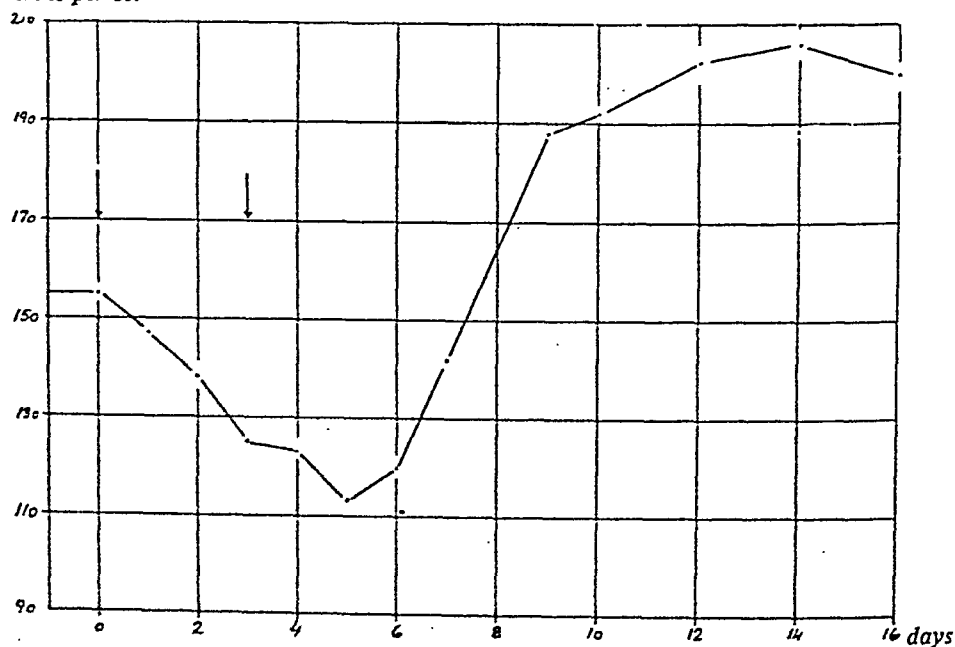


Fig. 7. Antithrombin content of the blood in chloroform poisoning.

→ = subcutaneous injection of 1 cc. of chloroform.

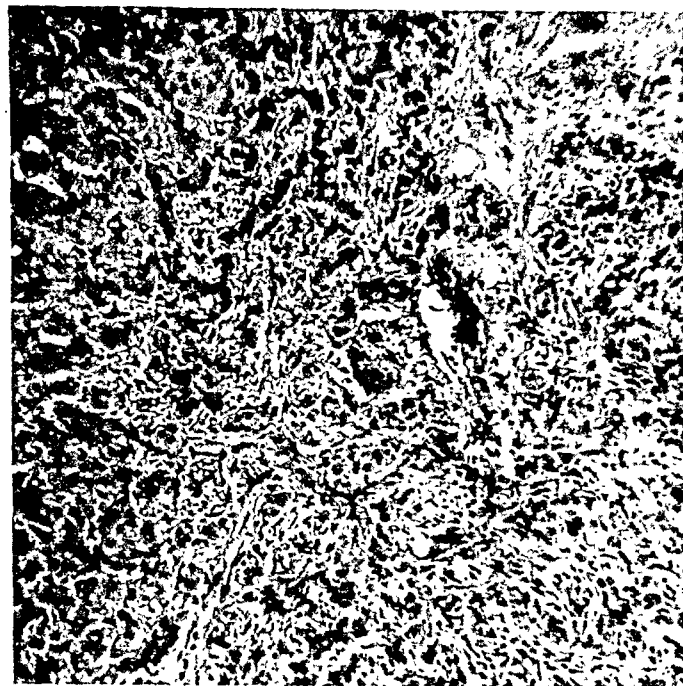


Fig. 8. Liver of a rabbit (No. 51) 18 days after ligation of the common bile duct.

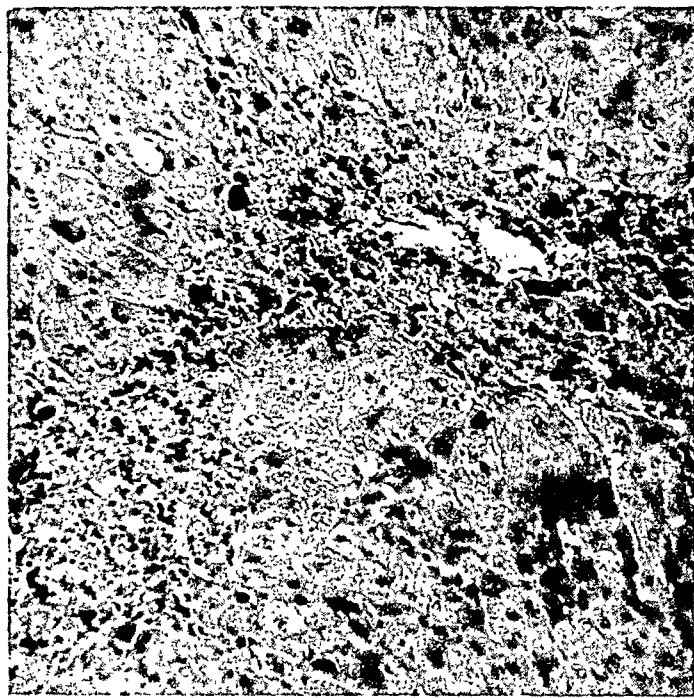


Fig. 9. Liver of a rabbit (No. 22) after poisoning with chloroform (2 injections of 1 cc.).

Sections stained with hematoxylin-eosin. Microphotos magnif.  $\times 150$ .



a fall in the antithrombin content of the blood while injury to the liver in obstructive jaundice gives a rise in antithrombin, it does not seem very likely that the increase in antithrombin in obstructive jaundice should be due to liver injury, and the cause is rather to be looked for elsewhere, as will be pointed out later on.

While thus the rise in antithrombin does not appear to be brought about by injury to the liver, there is no more reason for assuming that the fall in antithrombin in poisoning should be due to this injury. Even though poisoning with chloroform and carbon tetrachloride on microscopic examination manifests itself most distinctly in the severe damage to the parenchyma of the liver, it has to be kept in mind that these poisons have a universal toxic effect. As far as that goes, then, the cause of the fall in antithrombin concentration under these conditions may just as well be due to injury of some organ other than the liver. As will be pointed out later on, particularly damage to the reticulo-endothelial system is here to be taken into consideration.

### *Recapitulation.*

- A. 1. A review is given of the results arrived at in previous investigations on the antithrombin content of the blood in obstructive jaundice.
2. In the writer's investigations, experimental obstructive jaundice in rabbits is shown to be followed by a rise in antithrombin; details concerning this finding are discussed.
3. It is pointed out that the antithrombin reactions in obstructive jaundice are very similar to those found on immunization.
4. In one case the rise is found to stop and the antithrombin concentration is seen to fall off again. On autopsy this phenomenon was found to be due to the fact that the ligature had slipped off so that the free passage of the bile was reestablished.
- B. 1. A review is given of previous work dealing with the antithrombin content in liver injury.

2. In the writer's experiments, the liver is injured by injection of chloroform and carbon tetrachloride. In all the experiments this treatment gives a fall in the antithrombin content.
3. When the injections are discontinued, the antithrombin content is again increased rapidly, not only to a normal level but even considerably higher.
4. It is pointed out that as injury to the liver in experimental obstructive jaundice is associated with a rise in antithrombin concentration and in poisoning with a fall, there can hardly be any relation between the functional capacity of the liver and the antithrombin content of the blood.

## Chapter VII.

# VARIATIONS IN THE ANTITHROMBIN CONTENT OF THE BLOOD ON INTRAVENOUS INJECTION OF INDIA INK

As it had proved possible by intravenous injection of solutions of high-molecular substances without antigenic character to obtain the same antithrombin reactions as by immunization with protein solutions, the idea suggested itself to try whether injection of unspecific corpuscular substances might also give the reaction specific for immunization with suspensions.

For this purpose I have investigated the effect of intravenous injection of India ink, employing the commercial »Günther & Wagners Pelikan Perlтусch«.

The employment of this product ensured a constant uniformity of the injected preparation with regard to the size of the particles and the number of particles per cc.—qualities that would be difficult to duplicate if I were to prepare the India ink myself by grinding. On the other hand, the commercial preparation contains various by-products added for stabilization of the suspension, and thus it was possible that these substances might interfere with the biological processes here concerned. On comparison between the effect of Günther & Wagner's India ink and that of a suspension prepared by grinding India ink in 0.9 per cent NaCl no difference could be demonstrated, (see Table 27), and the advantage of working with a uniform preparation seemed therefore to counterbalance the drawbacks mentioned.

On intravenous injection of India ink, however, the effect turned out to be quite different from what was expected.

Thus injection of 0.1 cc. of India ink per kg. was followed



in normal rabbits by a sharp fall in the antithrombin concentration of the blood amounting to about 20 per cent. This fall was only of brief duration, however, and after 1 hour the antithrombin content was again normal. On repetition of the injection

*Antithrombin  
units per cc.*

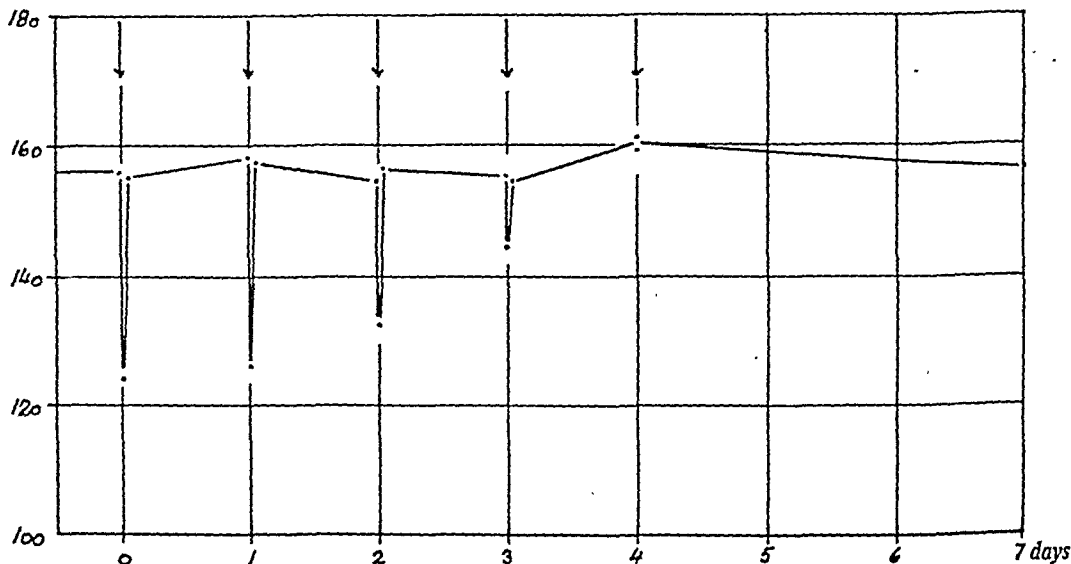


Fig. 10. Antithrombin concentration of the blood on repeated intravenous injection of 0.1 cc. of India ink per kg.

tion, for instance, next day, a similar brief fall appeared in the antithrombin content. On further continuation of these India ink injections, however, the fall in antithrombin content was found to decrease, and after the 5th injection there was no fall whatever. After 5 such injections, given at intervals of 24 hours, the antithrombin content was still normal. An example of these findings is shown in Fig. 10.

In the case presented in Fig. 10 the India ink injections were given at intervals of 24 hours, and prior to injection the dose of India ink, 0.1 cc. per kg. was diluted with 0.9 per cent NaCl to make 2 cc. The results obtained in 6 experiments of this kind are recorded in Table 17.

The duration of the fall in antithrombin after the individual India ink injections was not more than 1 hour—as is seen from Table 18.

*Table 17. Antithrombin Content on Repeated Intravenous Injections of Small Amounts of India Ink.*

Rabbit No.	Antithrombin content									
	Before 1' inj.	After 1' inj.	Before 2' inj.	After 2' inj.	Before 3' inj.	After 3' inj.	Before 4' inj.	After 4' inj.	Before 5' inj.	After 5' inj.
21	155	120	155	116	164	140	157	148	157	157
39	155	121	156	128	156	129	157	145	161	162
55	153	117	152	123	150	125	155	133	158	160
69	156	123	156	125	159	139	154	142	156	156
70	157	126	156	123	156	135	156	139	159	157
80	156	124	158	126	154	132	155	144	161	159

*Table 18. Duration of the Fall in Antithrombin Content on Injection of Small Doses of India Ink.*

Rabbit No.	Antithrombin content					
	Before 1' inj.	20 min. after 1' inj.	1 hour after 1' inj.	Before 2' inj.	20 min. after 2' inj.	1 hour after 2' inj.
69	156	123	155	156	125	156
70	157	126	156	156	123	157
80	156	124	155	158	126	157

Intravenous injection of larger amounts of India ink—for instance, 0.5 cc. per kg.—resulted in a sharp fall in the antithrombin content amounting to about 20 per cent, just as after injection of a small dose. But, while the effect of the small injection was only brief, the effect of the large injection was protracted, so that the antithrombin concentration of the blood now stayed at the low level for several weeks.

Repeated injection of 0.5 cc. of India ink per kg. or larger single doses—even so large (about 2 cc. per kg.) that they caused the death of the animal from intravascular coagulation—had apparently no greater effect than one injection of 0.5 cc. per kg. A survey of the findings in these experiments is given in Table 19.

As a curiosity it may be mentioned that as long as 20 minutes after injection of merely 0.5 cc. of India ink per kg. the plasma was often quite black from the India ink.

*Table 19. Antithrombin Content on Intravenous Injection of Large Amounts of India Ink.*

Rabbit No.	Amount of India ink injected	Antithrombin content		
		Before inj.	After inj.	4 weeks after inj.
9	1 inj. of 0.5 cc. per kg.	157	117	116
63	-	154	121	
26	2 injs. of 0.5 cc. per kg. at intervals of 24 hours	155	112	
24	3 injs. of 0.5 cc. per kg. at intervals of 24 hours	155	117	123
58	-	154	124	
59	4 injs. of 0.5 cc. per kg. at intervals of 24 hours	155	122	
73	1 inj. of 1 cc. per kg.	157	129	
18	1 inj. of 2 cc. per kg.	158	124	

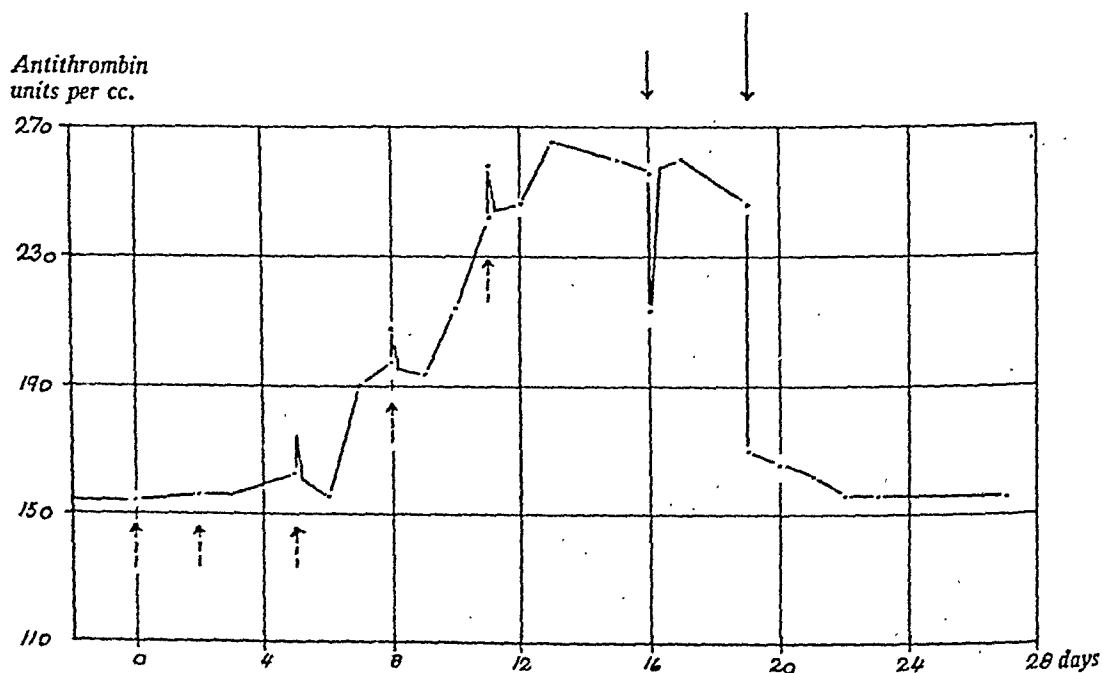


Fig. 11. Fall in the antithrombin content of the blood on injection of 0.1 cc. and 0.5 cc. of India ink per kg. when the antithrombin concentration beforehand is maximal on account of immunization.

---→ = injection of 2 cc. of edestin solution, intravenously.

→ = injection of 0.1 cc. of India ink per kg.

—→ = injection of 0.5 cc. of India ink per kg.

As injection of India ink thus had a marked effect on the normal antithrombin concentration of the blood, it would be interesting to see what the effect might be when the antithrombin concentration had been brought up to a maximal level, for instance, by immunization.

Rabbits in which this was the case were therefore given intravenous injections of India ink, first in a dosage of 0.1 cc. per kg. intravenously, just as in the experiments with normal rabbits. This injection resulted in a sharp fall, amounting to about 30–40 antithrombin units, that is, a considerably larger fall than seen in the normal rabbits, where it was only about 25 antithrombin units. In the course of 1 hour the antithrombin concentration rose again to the initial level. An example of these findings is given in Fig. 11.

The results obtained in 5 cases of this kind are given in Table 20.

*Table 20. Effect of Intravenous Injection of 0.1 cc. of India Ink per kg. in Rabbits with a Maximal Antithrombin Content of the Blood.*

Rabbit No.	Antithrombin content before inj.	Antithrombin content 20 min. after inj.	Antithrombin content 1 hour after inj.	Antithrombin content 24 hours after inj.
20	256	217		260
31	246	198	246	248
32	248	212	238	250
33	242	196	238	244
34	246	178	244	246

On the other hand, when large amounts of India ink were injected—for instance, 0.5 cc per kg.—the result was an abrupt fall in the antithrombin concentration down to a normal level, and this was not followed subsequently by any rise, but the antithrombin content kept constantly at this level. In one case (rabbit No. 20) the abrupt fall did not quite reach the normal level, but only 170; still, during the following days the fall continued slowly to a normal level. Repeated injections or larger doses had no additional effect on the antithrombin content beyond that here described, as in every instance the anti-

thrombin concentration fell off only to a normal level and could not be reduced further.

When these experiments were repeated on animals in which the antithrombin content of the blood was maximal from the primary rise in anaphylactic shock or from obstructive jaundice, the result was exactly the same. Here, also injection of large doses of India ink produced a sharp and lasting fall to normal level. The results of these experiments are recorded in Table 21.

*Table 21. Antithrombin Content on Intravenous Injection of Large Amounts of India Ink.*

Rabbit No.	Antithrombin maximum due to	Amount of India ink injected	Antithrombin content		
			Before inj.	After inj.	5 days after inj.
21	Immunization	1 inj. of 0.5 cc. per kg.	246	172	156
48	-	-	234	154	156
35	-	1 inj. of 1 cc. per kg., and 5 days later 1 inj. of 0.5 cc. per kg.	200	156	159
36	-	-	205	156	156
37	-	-	202	154	157
49	Obstructive jaundice	1 inj. of 0.5 cc. per kg.	250	156	
50	-	-	248	153	
51	-	1 inj. of 1 cc. per kg.	254	164	
40	Anaphylactic shock	1 inj. of 0.5 cc. per kg.	236	155	156
41	-	-	236	157	
42	-	1 inj. of 1 cc. per kg.	240	166	157

Besides the immediate direct effect of the India ink injection on the antithrombin content, it seemed to be of interest also to learn whether such injections might give other disturbances.

For this purpose I tried if it might be possible with India ink to block the antithrombin formation under conditions which otherwise gave rise to an increase in antithrombin.

By administration of the India ink in several small injections, as demonstrated above, it is possible to inject fairly large amounts of India ink intravenously apparently without any lasting change in the antithrombin content.

When now—after the animal had been given 5 injections of 0.1 cc. of India ink per kg.—an attempt was made to produce a rise in the antithrombin content by immunization in the usual manner, this proved no longer to be practicable. Immunization produced now neither the general rise in antithrombin content nor the small characteristic brief reactions in response to the later injections. After the immunization the antithrombin concentration was still normal. Thus the India ink had completely blocked the antithrombin formation over the normal value.

The fact that this is possible proves that the increase in antithrombin during immunization has nothing to do with the antibody formation. For, even though opinions are still divergent as to the effect of India ink injection on the antibody formation, at any rate it is known with certainty that this treatment at the most may delay the antibody formation, but will never quite prevent it (cf. KREYBERG<sup>78</sup>, ROBERTS<sup>112</sup>, CANON, BAER, SULLIVAN & WEBSTER<sup>25</sup>, JELIN, ROSENBLATT & BRIN<sup>74</sup>).

When India ink blockade was applied in the same manner immediately before or just after ligation of the common bile duct it likewise prevented any antithrombin reaction, and the antithrombin concentration kept constantly at a normal level up to the death of the animal.

When the blockade was applied to rabbits which 4 weeks previously had been sensitized in the usual manner with protein solutions, and an attempt then was made to produce anaphylactic shock by reinjection of the antigen, the result was likewise a complete absence of any antithrombin reaction, as there was neither a primary rise nor a secondary. Also in these cases the India ink injections completely blocked the antithrombin formation.

The results of these experiments are recorded in Tables 22, 23 and 24.

When 4 weeks after such a blockade an attempt was made to produce a rise in the antithrombin content by performing immunization or obstructive jaundice, the result was now an increase in antithrombin; but this was considerably less than seen in the normal rabbits. Thus rabbit No. 23 showed on

Table 22. *Effect of India Ink Blockade on the Rise in Antithrombin Content during Immunization.*

Rabbit No.	Blockade with 5 injs. of 0.1 cc. of India ink per kg.	Antithrombin content after injs. of India ink	Immunization with 5 injs. of 2 cc. in 11 days with	Antithrombin content		
				Before immunization	After immunization	5 days after immunization
21	-	157	5 percent edestin solution Bovine BORDET plasma	155	157	156
23	-	158		156	156	155
29	-	160		160	158	156
69	-	156		158	160	158
70	-	157		156	159	158

Table 23. *Effect of India Ink Blockade on the Rise in Antithrombin Content in Experimental Obstructive Jaundice.*

Rabbit No.	Blockade with 5 injs. of 0.1 cc. of India ink per kg.	Antithrombin content after injs. of India ink.	Experimental obstr. jaundice by ligation of common bile duct	Antithrombin content				
				Before operation	7 days after oper.	14 days after oper.	21 days after oper.	30 days after oper.
25	-	156	-	157	158	158		
27	-	155	-	155	155	158	156	157
30	-	156	-	157	155	156	154	
38	-	156	-	156	156	161		159
39	-	162	-	160	159	154	154	

Table 24. *Effect of India Ink Blockade on the Rise in Antithrombin Concentration during and after Anaphylactic Shock.*

Rabbit No.	Sensitized with	Daily inj. of India ink 0.1 cc. per kg. for 5 days before shock	Shock-provoking injection	Antithrombin content		
				Before shock	20 min. after inj.	24 hrs. after inj.
44	Bovine BORDET plasma	-	2 cc. bovine BORDET plasma	158	156	155
82	-	-	-	155	157	151
83	-	-	-	161	157	153
84	-	-	-	161	161	159

immunization a rise from 156 antithrombin units per cc. to 185; and in experimental obstructive jaundice in rabbit No. 29 the antithrombin concentration rose in 3 weeks from 156 to 195 antithrombin units. So the effect of the blockade was now subsiding.

In view of the relatively brief effectivity of the blockade it seems rather striking that experimental obstructive jaundice after the India ink blockade was not followed even by a slight rise in antithrombin, although several of the animals survived the operation for up to 4 weeks. According to the above-mentioned experiments, the blockade should not be effective towards the end of the lifetime of these animals. The failure of these animals to show any antithrombin reaction is probably due to the circumstance that 3-4 weeks after the operation they were extremely emaciated and in poor condition.

As a supplement to these studies on rabbits, I tried in the case of one cat to see how the antithrombin concentration in this animal would behave in response to an intravenous injection of 0.5 cc. of India ink per kg.

The injection was given under ether anaesthesia into the left femoral vein. The antithrombin formation was measured before the injection and several times after.

In immediate response to the injection there was an abrupt rise in the antithrombin content, amounting to about 30 antithrombin units per cc., but this increase was soon followed by a fall, the antithrombin concentration returning to a normal level in four hours. Instead of stopping at the initial level, however, the fall continued for a couple of hours, till it reached a value about 20 per cent below the normal level. After this, the antithrombin content kept constant at this low level throughout the observation period, which was 24 hours. These features are presented graphically in Fig. 12.

So, apart from a brief, relatively slight rise in antithrombin in response to the India ink injections, the final result in the cat was the same as observed in the rabbits, namely: a protracted decrease of about 20 per cent in the antithrombin content.

According to investigations reported by KLEIN & LEVISON<sup>77</sup>,



TURCU<sup>124</sup>, and by DUDGEON & GOADBY<sup>38</sup>, injection of India ink produces a number of widely different changes in the blood as, for instance: hypoproteinæmia, hypocholesterinæmia, thrombopenia, changes in pH, and increase in blood sugar.

Antithrombin  
units per cc.

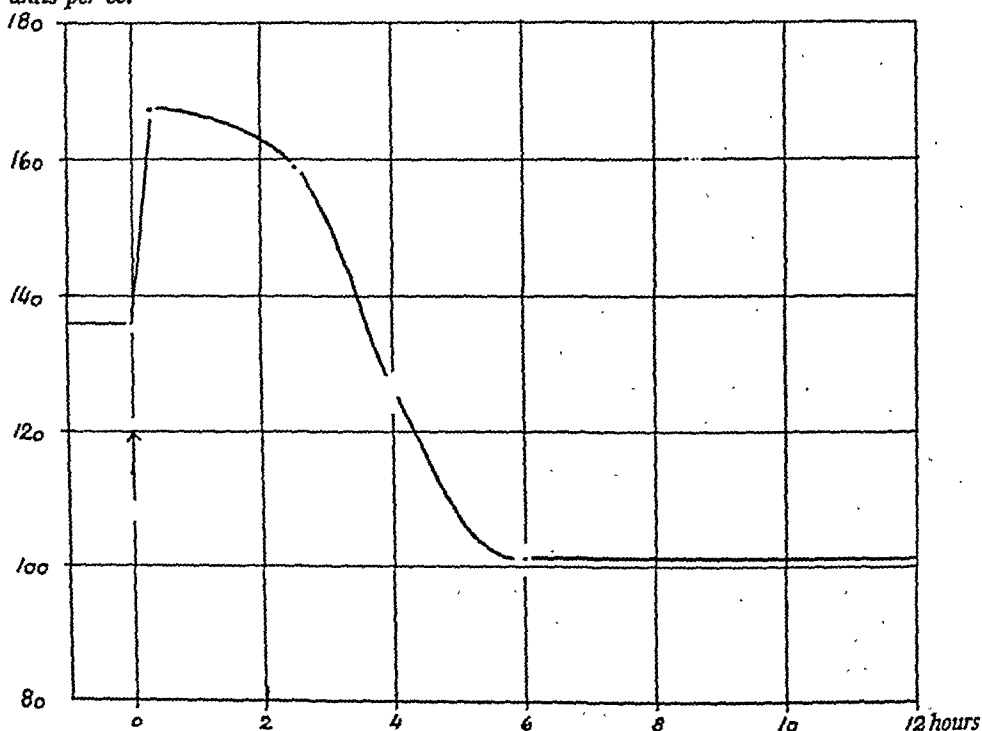


Fig. 12. Antithrombin content of the blood in a cat after injection of 0.5 cc. of India ink per kg. at  $\uparrow$ .

Before I commenced to look among these possibilities for an explanation of the effect of India ink upon the antithrombin concentration, it was natural, therefore, first to see whether it might be possible to produce a corresponding phenomenon in vitro and eventually approach an explanation in this way.

As, according to JANCOS<sup>71</sup>, the addition of India ink to plasma results in flocculation of the fibrinogen round the granules of India ink, and as this, according to the same author, takes place also in vivo, it seemed conceivable that the effect of India ink on the antithrombin content might merely be due to the circumstance that the antithrombin was adsorbed and

removed during flocculation. To settle this question, the first step was therefore to add India ink to plasma in vitro. The amount of India ink added was 1 drop per cc. of plasma, and the mixture was incubated for 15 minutes at 37°.

As observed by JANCSO, the result was a marked flocculation of fibrinogen round the India ink granules, so that the plasma after centrifuging was almost perfectly clear. In spite of this, there was no change in the antithrombin content of the plasma. So the India ink effect was not due to a simple adsorption of antithrombin to the flocculated fibrinogen.

But, when the same amount of India ink was added to citrated blood before the corpuscles were separated from the plasma by centrifuging, and the mixture was otherwise treated as described above, the result was a large fall in antithrombin content. The findings in this experiment are recorded in Table 25.

*Table 25. Effect of Addition of India Ink to Plasma and Citrated Blood.*

Rabbit No.	Initial antithrombin content	Addition of India ink to	Antithrombin content after addition of India ink
40	234	plasma	234
49	254	-	254
51	248	-	242
54	246	-	246
31	170	citrated blood	145
32	163	-	135
67	254	-	164
83	242	-	161
84	244	-	158

That India ink had no effect on citrated plasma, whereas it decreased the antithrombin content of citrated blood seemed to suggest that it was the influence of India ink upon the blood cells that produced the fall in antithrombin content. In order to settle this question conclusively the following experiment was performed:

4 cc. of citrated blood was taken from each of 4 rabbits.

The corpuscles were separated by centrifuging, washed 5 times by repeated suspension in 0.9 per cent NaCl followed by centrifuging. After the last centrifuging the corpuscles were again suspended in sufficient 0.9 per cent NaCl to make a total volume of 4 cc. To each of these suspensions 2 drops of India ink were added, and then the mixtures were incubated at 37° for 15 minutes. After this, the corpuscles were again separated by centrifuging, washed 3 times, and were now added to their respective plasma. After incubation of these "specimens" at 37° for 15 minutes, the corpuscles were again removed by centrifuging, and the antithrombin content of the plasma was determined as usual. All four specimens showed that after the blood corpuscles had been in contact with the India ink they lowered the antithrombin content of the plasma. The findings in this experiment are recorded in Table 26.

*Table 26. Effect of India Ink-treated Blood Corpuscles on the Antithrombin Content of Citrated Plasma.*

Rabbit No.	Initial antithrombin content of plasma	Antithrombin content after addition of India ink-treated corpuscles
12	160	129
68	162	133
67	258	155
82	236	152

In addition, a control test (rabbit No. 83) was made in exactly the same manner, but without India ink treatment of the corpuscles. This, too, showed a fall in antithrombin content of the plasma (from 246 to 234). This fall, I think, is due to the dilution of the plasma by the small amount of water present between the corpuscles after their washing, in spite of the centrifuging. But, as the figures show, this fall was very slight.

From these results it is plainly evident that the India ink effect on the antithrombin content of the plasma is due to the influence of the India ink upon the corpuscles.

In order to ascertain whether this effect of India ink might

possibly be due to the by-products present in the commercial product, I then made an India ink preparation by grinding solid India ink in 0.9 per cent NaCl. This preparation was added to citrated blood, and after incubation and removal of the corpuscles by centrifuging the antithrombin content of the plasma was measured. This India ink preparation was found to lower the antithrombin content to the same extent as did Günther & Wagner's India ink. So the by-products had no measurable influence in this respect.

The results in this experiment are recorded in Table 27.

*Table 27. Comparison of the Effect on the Antithrombin Content obtained with Günther & Wagner's India Ink and with a Hand-ground India Ink Suspension.*

Rabbit No.	Antithrombin content of untreated plasma	Antithrombin after treatment with hand-ground India ink	Antithrombin after treatment with G. & W.'s India ink
81	182	156	158
82	192	155	156
83	242	163	161
84	244	154	158

How the exposure of the blood corpuscles to India ink may result in the fall in the antithrombin content of the plasma will be discussed later on.

These findings explain in part the fall in the antithrombin content of the blood observed after injection of India ink. The questions still remain, however, why the effect of a small dose is only of brief duration, why repeated injections of small doses gradually lose their effect, and why injection of a large dose of India ink in normal rabbits produces merely a fall of 25-30 antithrombin units per cc. while the same injection in animals with a maximal antithrombin concentration of the blood gives a fall of about 100 antithrombin units per cc.

Regarding the first of these questions, the temporary fall in antithrombin produced by the small India ink injections may indicate that the antithrombin is only temporarily affected by

or combined with the blood corpuscles and is soon liberated again; or it indicates that the fall in antithrombin content resulting from this treatment immediately is compensated by an increased antithrombin production.

Assuming the first of these possibilities, the decreasing effect from repeated small injections will probably be due to the circumstance that the blood corpuscles gradually get "accustomed" to the India ink and become resistant against its effect, so that they no longer combine with the antithrombin even in the presence of India ink. If the cause is to be found in the other possibility, however, the explanation of the phenomenon is rather to be searched for in the circumstance that the blood corpuscles gradually are affected maximally, becoming unable to combine with or act upon additional amounts of antithrombin even when the injection of India ink is continued. But this maximal influence must be effectuated before the antithrombin formation is blocked completely by the India ink, as otherwise the final result would have been that the antithrombin level of the blood after repeated injections of small doses of India ink would be lower than normally—something which the experiments show is not the case. As 5 injections of 0.1 cc. of India ink per kg. gave a complete blockade of the antithrombin formation, while the 4th. injection still gave a fall in antithrombin of about 10 per cent, the maximal effect of the India ink treatment upon the blood corpuscles should appear simultaneously with the complete blockade of the antithrombin formation. Even though this be possible, I think it seems improbable, and I am inclined therefore to attribute the effect of the India ink injections to a temporary combination of the antithrombin with the blood corpuscles.

Also a third possibility might seem conceivable, namely that the antithrombin through the repeated injections of India ink is undergoing such changes that it no longer is susceptible to the influence of the blood corpuscles. This is not the case, however, as is evident from the following experiments.

For 5 days 4 rabbits were given a daily injection of 0.1 cc. of India ink, and 24 hours after the last injection a suitable amount of citrated blood was withdrawn. The plasma of this

blood was then mixed with a corresponding amount of blood corpuscles of normal animals (Nos. 84 and 67), and one drop of India ink per cc. was added to this mixture. After incubation for 15 minutes at 37°, followed by centrifuging, the antithrombin content of the 4 specimens of plasma was measured, and on comparison with the same plasma without addition of blood corpuscles and India ink, the antithrombin content was found to have decreased even rather considerably through the process mentioned. The findings are given in Table 28.

*Table 28. Antithrombin Content of Plasma obtained after Five Injections of 0.1 cc. of India Ink per kg., and then treated by Addition of Normal Blood Corpuscles + India Ink.*

Rabbit No.	Initial antithrombin content	Antithrombin content after 5 injs. of 0.1 cc. of India ink per kg.	Antithrombin content after addition of corpuscles + India ink
75	160	157	133
80	156	156	132
82	155	151	113
83	156	153	119

When this experiment was reversed, and the blood corpuscles from rabbits Nos. 75, 80, 82 and 83 were added to fresh plasma, followed by the addition of India ink to these mixtures, there was no fall in the antithrombin content of the plasma. This indicates that the absence of India ink effect from repeated small injections of this substance is due to changes in the blood corpuscles. This experiment was carried out with plasma from rabbits Nos. 84 and 67. The antithrombin content of these plasmas was respectively 244 and 153. After the addition of India ink and blood corpuscles from rabbits Nos. 75, 80, 82 and 83 taken immediately after the 5 India ink injections of 0.1 cc. per kg. the antithrombin content of the two plasmas was found to be 246 and 248 in the case of No. 84, and 151 and 155 in the case of No. 67.

In analogy to the explanation of the effect of the small India ink injections, the effect of the large doses may be explained so,

that the influence of the India ink upon the corpuscles now is sufficiently large to make their combination with the antithrombin permanent. At the same time, however, a dose of India ink of 0.5 cc. per kg. or more gives complete blockade of the antithrombin formation, so that the protracted effect may conceivably be ascribable to this blockade too.

The last question as to why large doses of India ink in normal animals give a considerably smaller fall in the antithrombin concentration of the blood than observed in animals with a maximal antithrombin concentration will be dealt with in the next chapter.

As several authors have attributed the thrombin-inactivating property of the blood to enzymatic inhibition by means of the plasma proteins, and as the injection of India ink is claimed to exert its influence just on the protein content of the blood, I examined in 3 cases whether an injection of 0.5 cc. of India ink per kg., which gives a maximal fall in antithrombin, would have any particular effect on the plasma proteins. The total nitrogen content of the plasma was taken as a measure of its protein content; it was determined by means of the micro-KJELDAHL procedure.

In all three cases the injection of 0.5 cc. of India ink per kg. was found not to diminish the protein content. So the fall in antithrombin content resulting from injection of India ink is independent of any hypoproteinæmia that may arise from the India ink injection.

The findings in this experiment are given in Table 29\*).

*Table 29. Nitrogen Content of Citrated Plasma before and after Injection of India Ink.*

Rabbit No.	N content of normal citrate plasma	N content of plasma after inj. of India ink
71	7.75 mg. per cc.	7.82 mg. per cc.
72	9.10 - - -	9.62 - - -
73	7.74 - - -	8.50 - - -

\*) That the injections of India ink give a little rise of the nitrogen content, must be due to a concentration of the blood resulting from shock produced by the injections.

*Recapitulation.*

1. Intravenous injection of a small dose of India ink produces a brief fall in the antithrombin concentration of the blood amounting to about 20 per cent.
2. On repeated injection of small doses of India ink this effect on the antithrombin content ceases gradually.
3. Injection of a large dose of India ink produces a lasting fall in the antithrombin content amounting to about 20 per cent, and even repeated injections or maximal doses of India ink have no additional effect in this respect.
4. Injection of a large dose of India ink in animals with a maximal antithrombin content of the blood produces a sharp fall to normal level. This holds true irrespective of the method by which the antithrombin content has been brought up to a maximum.
5. 5 injections of 0.1 cc. of India ink are sufficient for complete blockade of the antithrombin formation during immunization, anaphylactic shock and obstructive jaundice.
6. Injection of large doses of India ink in a cat shows a brief primary rise in antithrombin, but the final result is the same as observed in the rabbits—a protracted decrease amounting to about 20 per cent.
7. This effect of India ink is demonstrated to be due to its influence upon the blood corpuscles.
8. The India ink effect is discussed.
9. Intravenous injection of 0.5 cc. of India ink per kg. is demonstrated not to produce any decrease in the protein content of the plasma.



## Chapter VIII.

# THE ANTITHROMBIN CONTENT ON LOSS OF BLOOD

It was found by earlier authors that the antithrombin content of the blood may be decreased not only by injection of India ink but also by bleeding. This phenomenon was first observed by DRINKER & DRINKER<sup>37</sup> (1917) who submitted rabbits and cats to withdrawal of large amounts of blood, while the antithrombin content of the blood was examined at the same time after HOWELL's method. In this way they thought they were able to demonstrate that the loss of blood was associated with a fall in antithrombin content, and this fall appeared to be considerably larger than would be expected for the dilution of the blood following the "hæmorrhage"—the more so as this dilution resulted chiefly from an increased afflux of lymph, which according to HOWELL contained just as much antithrombin as the blood. The authors therefore assumed this fall in antithrombin to be produced as an active protective measure on the part of the organism possibly aimed to bring about the shortened coagulation time of the blood resulting from the bleeding.

As I have not been able to find in the literature any other report concerning this question, I found it appropriate to take it up for further study.

### *Writer's Investigations.*

In my experiments 4 rabbits and 2 cats were bled. In one case (rabbit No. 4) the blood was withdrawn from one of the large veins of the neck by means of a Record syringe, in the other cases from the carotid artery through a glass cannula.

The operation was performed under ether anæsthesia. The

first 3 cc. of the outflowing blood were used for measuring the antithrombin content prior to the loss of blood.

Owing to the fall in blood pressure produced by the bleeding, it became difficult to follow in the rabbits the antithrombin content through samples of blood taken from the ear veins. In these animals, as well as in the cats, therefore, the samples of blood required after the bleeding were taken from the arterial stump by means of a Record syringe equipped with a hypodermic needle. In order to exclude the possibility that the blood in the closed arterial stump should not be mixed properly with the rest of the blood and thus give an untrue picture of the antithrombin content, in several instances control determinations were made on blood withdrawn from one of the large veins of the neck. In every instance the values obtained in this way agreed with the determinations on the arterial blood. So the pulsating movement of the blood in the arterial stump must have been sufficient to mix it thoroughly with the rest of the blood.

In the animals in which the blood was taken from the artery, the amount of blood withdrawn would be about 30 per cent of the total (this being reckoned as equal to about 5.5 per cent of the weight of the animal). In the case of the rabbit which was bled from the vein and which constituted the first orientating experiment, the amount of blood withdrawn was somewhat smaller.

By measuring the antithrombin content of the blood at short intervals after the bleeding, I found it to be falling off rapidly, reaching a minimum 10-15 minutes after the bleeding, then it kept constant for some length of time, whereafter it began slowly to rise again. A normal level was reached about 48 hours after the bleeding.

In every instance this fall in antithrombin content was only what could be expected from the dilution of the blood. In order to establish more precisely the fall in antithrombin content in relation to the dilution of the blood, in 4 cases the nitrogen content was determined on the plasma simultaneously with the determination of the antithrombin content, and in every in-

stance the variations in the nitrogen content were found to be of the same magnitude as the fall in the antithrombin content.

The nitrogen determinations were carried out by means of the micro-KJELDAHL procedure.

In one case the hæmoglobin content of the blood was examined simultaneously with the antithrombin determinations, and the fall in hæmoglobin percentage was found to be practically identical with the fall in antithrombin content. The hæmoglobin percentage was determined on each sample of blood by means of 4 independent measurements with SAHLI's apparatus.

A survey of the findings in this experiment is given in Table 30.

My findings are thus seen not to confirm the observations reported by DRINKER & DRINKER—that the fall in antithrombin after bleeding should be larger than would be expected when the dilution of the blood is taken into consideration. So the amount of fluid poured out into the blood stream to compensate

*Table 30. Antithrombin Content on Loss of Blood.*

Animal No.	Weight in kg.	Amount of blood withdrawn in cc.	Antithrombin content			Mg. N per cc. before bleeding	Mg. N per cc. after bleeding	Fall in N in per cent	Antithrombin content	
			Before bleeding	After bleeding	Fall in per cent				24 hours after bleeding	48 hours after bleeding
Rabbit 1	2.8	45	156	126	19.2	8.49	6.07	28.6		154
Rabbit 2	2.6	40	164	124	24.4	8.85	6.62	25.2	134	164
Rabbit 4	2.85	35	157	139	11.5				131	163
Rabbit 5	2.7	45	160	110	31.2					152
Cat 13	2.5	60	142	94	33.8	9.36	7.36	21.0	102	
Cat 14	2.0	45	134	108	19.4	9.17	7.69	16.8	122	

the loss of blood can probably contain only a relatively small amount of antithrombin.

It looks then as if the fall in the antithrombin content on bleeding may be explained as due exclusively to the dilution of the blood and not, as assumed by DRINKER & DRINKER, to the interference of specific measures on the part of the organism against further loss of blood.

*Recapitulation.*

1. According to observations reported by DRINKER & DRINKER, the fall in antithrombin content associated with a large loss of blood should be considerably larger than was to be expected in view of the dilution of the blood occurring after hæmorrhage. My findings in 6 animals show, however, that this is not the case. In every instance the fall in antithrombin content has been in accordance with the fall in the total nitrogen content of the plasma and in the hæmoglobin percentage of the blood, so that it may be ascribed exclusively to the dilution of the blood.

## Chapter IX.

# STUDIES ON THE IDENTITY OF ANTITHROMBIN

As it was possible by injection of India ink and by bleeding to produce a fall in the antithrombin content, and as the India ink injections besides lowering the antithrombin content cause also blockade of its formation, the idea suggested itself, by a combination of India ink injections and bleeding to try completely to remove the antithrombin from the blood.

With this aim 5 rabbits were given an injection of 0.5 cc. of India ink per kg., which, as demonstrated previously, gave a lasting fall in the antithrombin content of about 20 per cent and a complete blockade of the antithrombin formation. 24-48 hours later, a large amount of blood was withdrawn from one of the common carotids. As was to be expected, this caused an additional fall in the antithrombin content. It was found, however, that just as in normal animals, this last fall in antithrombin content was followed by a rise, and in 48 hours the antithrombin content reached again the level it attained after the injection of India ink. The rise stopped at this level, however, and then the antithrombin content kept constant for a considerable length of time.

So the injections of India ink cannot block the rise in antithrombin content under these conditions.

The results obtained in these experiments are seen in Fig. 13 and Table 31.

On recapitulation of the various experimental results the following features may be remarked:

- 1) Immunization, anaphylactic shock and obstructive jaundice produce a rise in the antithrombin content of the blood, and this rise is in every instance of the same magnitude. The rise may be prevented completely by intravenous injection of

India ink. If the rise in antithrombin content due to one of the causes mentioned has taken place already, addition of India ink to the blood produces *in vivo* as well as *in vitro* a sharp

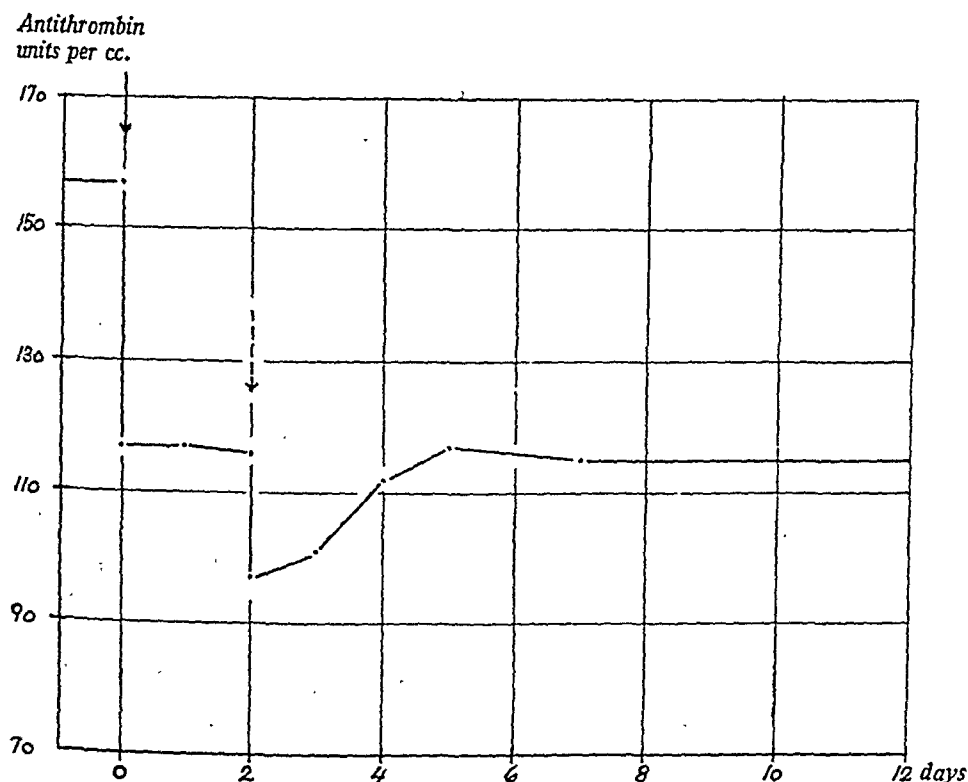


Fig. 13. Antithrombin content of the blood on intravenous injection of 0.5 cc. of India ink per kg. followed by bleeding.

—→ = intravenous injection of 0.5 cc. of India ink per kg.

---→ = bleeding.

Table 31. *Antithrombin Content on Intravenous Injection of India Ink and Subsequent Bleeding.*

Rabbit No.	Antithrombin content						
	Before inj.	After inj.	20 min. after bleeding	24 hours after bleeding	48 hours after bleeding	4 days after bleeding	11 days after bleeding
9	157	117	97	101	112	117	
24	155	117	98	97	127	131	123
71	154	130	93	95	132	135	132
72	154	134	99	102	134	131	131
73	157	129	97	104	133	130	129

fall in the antithrombin content down to a normal level. This is due to the influence of India ink upon the blood corpuscles. So the antithrombin formed on immunization, in anaphylactic shock and in obstructive jaundice behaves perfectly alike, so that it has to be regarded as the same substance.

2) Addition of India ink to normal blood in vivo as well as in vitro produces likewise a fall in antithrombin content, but this amounts only to about 20 per cent of the initial content. This fall is likewise due to the influence of India ink upon the blood corpuscles. When the injected dose of India ink is small, the reaction is only of brief duration. With a large dose, however, the reaction is protracted, probably due in part to a blockade of the antithrombin formation by the India ink. As addition of India ink to blood with a maximal antithrombin content produces a fall of 100 antithrombin units per cc. of plasma, while the same amount of India ink added to normal blood produces merely a fall of about 25 antithrombin units per cc. of plasma, it is obvious that the slight fall in the latter instance cannot be due to an incapacity of the India ink + blood corpuscles for combination with more antithrombin, and that the cause must be due to the circumstance that only 20 per cent of the normal antithrombin can be removed in this way.

Furthermore, when by injection of India ink the antithrombin content of the blood is lowered about 20 per cent and the animal is then bled, the India ink cannot prevent the resulting additional fall from being compensated by a rise in antithrombin content.

These findings appear to show that about 20 per cent of the normal antithrombin behaves as the antithrombin formed during immunization, in anaphylactic shock and in obstructive jaundice while the remaining 80 per cent differ fundamentally herefrom, as it is impossible by means of India ink to block its formation or to remove it.

Probably, therefore, the blood antithrombin consists of two components:

- 1) One component, which normally amounts to about 80 per cent of the total blood antithrombin. It appears to be rather constant and takes only a slight part, if any, in the rise

- produced during immunization, anaphylactic shock and obstructive jaundice. This component is not affected by injection of India ink. On dilution of the blood by bleeding, its concentration is decreased too; but this fall is compensated rapidly by a rise, regardless of any attempt at blockade.
- 2) Another component which normally makes up only 20 per cent of the total. Under various pathological conditions it can be increased to many times its initial value. This component can be removed completely from the blood by intravenous injection of India ink and, *in vitro*, by addition of India ink to the blood. Furthermore, it is possible in every instance by means of India ink to block its above-mentioned increase.

*Regarding the first of these components, which makes up the major part of the normal antithrombin content of the blood, it possibly—or even probably—is not a real substance but only identical with the unspecific (or specific) enzyme inhibition due to the plasma proteins.* The argumentation for the probability of the plasma proteins playing a part in the antithrombic activity of the blood has been reviewed already in Chapter III and will not be repeated here. But I want to call attention to the fact that the previous inaccurate measuring methods have not made it possible to demonstrate whether the fractionated blood proteins which have been shown to be of antithrombic character exert the entire antithrombic activity of the blood or merely 80 per cent of it.

As this component seems rather constant, moreover, under normal as well as pathological conditions, the objection to the proteins as the cause of the antithrombic activity of the blood is invalidated. This objection was based on the peculiar fact that the antithrombin content of the blood may vary greatly, whereas the concentration of the plasma albumins keeps constant. In this connection it is appropriate to recall the observation that injection of 0.5 cc. of India ink per kg., which gives a maximal fall in the antithrombin content; did not produce a fall of the protein content of the blood.

We have seen, however, that when the antithrombin content is maximal, on account of one of the aforementioned conditions,



the injection of India ink gives a fall in antithrombin only to a normal level, and not below this. This finding appears to indicate that also this component takes some minor part in the rise in antithrombin under discussion\*).

As to the other component, which normally makes up 20 per cent of the antithrombin content, the explanation is less obvious. Here I have considered in particular the question whether it might be heparin or, more correctly, heparin + co-factor. Below I shall review the findings that seem to confirm this view.

As mentioned in Chapter III, addition of heparin to plasma produces an increase in the antithrombic activity of the plasma. This is due to the circumstance that heparin together with an unknown thermolabile component present in the plasma forms a new independent complex which is able to neutralize the thrombin. ASTRUP & DARLING<sup>12</sup> found that when increasing amounts of heparin are added to that fraction of the plasma proteins which contains the chief amount of the cofactor; the antithrombic activity of this fraction increases gradually. This increase ceases at a certain heparin concentration, however, and further addition of heparin does not further increase the thrombin-inactivating capacity of the fraction. Accordingly, then, the cofactor is used up gradually on addition of heparin, and when it is used up completely, the antithrombic activity cannot be increased further on addition of heparin because, as mentioned before, heparin alone is inactive.

In order to ascertain whether these findings apply also to plasma, I made an experiment with addition of heparin in increasing amounts to fresh citrated plasma of rabbits. To a series of tubes containing 0.5 cc. of this plasma, 0.5 cc. of a heparin solution was added to each of these tubes—with a gradual increase in the concentration of the heparin solution. In the control test 0.9 per cent NaCl was added instead of heparin. The heparin preparation employed here was Heparin "Leo" with an activity of k 4-5 (ASTRUP & BEHRNTS JENSEN<sup>7</sup>), which corresponds to about 25,000 HOWELL units per gram. The solvent was 0.9 per cent NaCl.

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\*) See also p. 112.

After the addition of heparin and thorough mixing by shaking, the tubes were placed in a water-bath at  $37^{\circ}$  for 15 min. in order to make sure of a quantitative combination between heparin and the cofactor. After this the antithrombic activity of the mixture was measured in the usual manner, but without heat coagulation of the plasma.

This experiment showed, in accordance with the findings of ASTRUP & DARLING in their work with plasma fractions, that the increasing heparin content of the plasma produced an increasing antithrombic activity of the plasma—up to a certain maximum. When this maximum was reached it was impossible even with the largest doses of heparin to produce any additional increase in the thrombin-inactivating property of the plasma. But if a little fresh plasma was added to a plasma-heparin mixture containing heparin in excess, a new increase in the antithrombic activity was produced. So these experiments showed plainly that the cofactor for the antithrombic effect of heparin was used up gradually on addition of heparin. On addition of fresh plasma to the mixture, more cofactor was introduced into the mixture, and hence its antithrombic activity could increase again.

The rise in antithrombin resulting from addition of heparin was fairly proportional to the amount of heparin added when small amounts of heparin were used; but the proportionality varied somewhat from one animal to another.

With higher heparin concentrations the rise in antithrombin per unit of heparin added decreased, and the aforementioned maximum was reached with a heparin content of 10–15 mg. per cent in the plasma used.

Intravenous injection of 10–15 cc. of a 100 mg. per cent heparin solution in rabbits weighing 2500–3000 g. gave a rise in antithrombin similar to the rise observed on addition of heparin to plasma *in vitro*.

It is a most striking finding, however, that the maximum obtained by addition of heparin is exactly the same as that which appears when the rise in antithrombin is brought about by immunization, anaphylactic shock and obstructive jaundice, and

that the same physiological variations from one animal to another assert themselves here.

A survey of these findings is given in Table 32.

*Table 32. Antithrombin Content in Heparin Plasma.*

Rabbit No.	Antithrombin content in				
	Normal plasma	5 mg. per cent heparin plasma	10 mg. per cent heparin plasma	20 mg. per cent heparin plasma	40 mg. per cent heparin plasma
68	155	178	198	228	226
75	156	194	204	222	220
77	155	208	228	236	240
62	153	217	240	238	
39		209		260	
47				262	
46				262	258
78	157		212		

We have seen that when the maximum of antithrombin content is reached during immunization, additional injections of antigen give merely a slight and brief rise in antithrombin. Something similar is found here. When the antithrombin content has reached maximum from one of the aforementioned causes, no further rise of significance is obtained by intravenous injection of heparin or by addition of heparin to plasma in vitro, only a minor rise is found, amounting to about 10 per cent of the rise observed on addition of heparin to normal plasma. The findings in the experiment giving this result are recorded in Table 33.

In the first four rabbits the increased antithrombin value was due to immunization, in the last three to obstructive jaundice.

If the fibrinogen solution employed for the antithrombin determination contained the heparin cofactor, it must be in so small amounts that it could not influence the above-mentioned results. This is plainly evident from ASTRUP & DARLING'S<sup>12</sup> experiments. These authors showed that addition of heparin to serum gave no rise in the antithrombin content. This finding,

*Table 33. Amount of Antithrombin on Addition of Heparin to Plasma containing a maximum of Antithrombin.*

Rabbit No.	Antithrombin content before addition of heparin	Antithrombin content after addition of heparin (20 mg.) per cent
43	236	256
44	242	254
47	236	250
48	234	256
49	254	270
51	248	258
54	246	258

which I have been able to confirm, shows not only that serum contains no cofactor, but also that the cofactor content of our fibrinogen solution plays no rôle.

The antithrombin formed by addition of heparin behaves towards intravenous injections of India ink exactly in the same manner as antithrombin formed during immunization, in anaphylactic shock and in obstructive jaundice—under which conditions the injection of 0.5 cc. of India ink per kg. gave a sharp fall in the antithrombin content to a normal level. In the following experiment on rabbits with an increased antithrombin content of the blood due to intravenous injection of heparin, injection of India ink in the same dosage as above gave likewise a sharp fall in the antithrombin concentration to a normal level. The results of this experiment are recorded in Table 34.

*Table 34. Antithrombin Content of the Blood after Injections of Heparin and India Ink.*

Rabbit No.	Amount of heparin injected	Antithrombin content after inj. of heparin	Antithrombin after inj. of 0.5 cc. India ink
31	10 cc. of 100 mg. per cent heparin solution	234	155
32	-	220	151
74	20 cc. of 10 mg. per cent heparin solution	252	154

It is a striking observation that it was impossible also in this case to produce any larger fall in the antithrombin content;

for it would be difficult to imagine that there could have been any increase in the stable component. It may be, therefore, that here as well as during immunization, anaphylactic shock and obstructive jaundice this finding is due to the fact that the blood corpuscles + India ink are unable to combine with more antithrombin than corresponds to the fall observed. The preceding rise in antithrombin does not therefore involve the component which is refractory to the effect of India ink.

It is not only *in vivo*, however, that addition of India ink to heparin blood gives this result. Also *in vitro* does the antithrombin formed by addition of heparin disappear completely on addition of India ink—as illustrated by the experiment recorded in Table 35.

*Table 35. Fall in Antithrombin Content on Addition of India Ink to Heparin Blood.*

Rabbit No.	Antithrombin content of normal blood	Antithrombin content after addition of heparin	Antithrombin content in heparin blood after add. of India ink
75	156	220	154
77	155	240	160
68	155	226	153

FISCHER<sup>50</sup> has found that injury to the cell membrane of leucocytes—for instance, by chloroform—enables these cells to combine with large amounts of heparin. It seems natural, therefore, to assume that the exposure of the blood corpuscles to the action of India ink also implies injury to the corpuscles, and that it is for this reason that they are able to combine with antithrombin as well as heparin. If this be the case, the corpuscles should be able to combine with antithrombin also if they were injured in some way other than by India ink—for instance, by chloroform.

Various authors have reported that the antithrombin of plasma is destroyed by addition of chloroform. In order to ascertain if this holds true also, when only a very small amount of chloroform is added, an experiment was made in which one drop of chloroform was added to 2.5 cc. of a plasma containing a large

amount of antithrombin, and this gave no change in its antithrombin content. When, on the other hand, 1 drop of chloroform was added to 5 cc. of citrated blood, which likewise contained a large amount of antithrombin, the result was a sharp fall in the antithrombin content. Even though the addition of chloroform produced a slight hæmolysis, the resulting dilution is far from sufficient to explain the sharp fall in antithrombin, as is plainly evident from Table 36.

*Table 36. Fall in the Antithrombin Content of the Blood on Injuring the Corpuscles by Addition of Chloroform.*

Rabbit No.	Antithrombin content before addition of chloroform	Antithrombin content after addition of chloroform
81	248	160
82	250	155
67	231	159
64	188	156

These experiments show that the fall in antithrombin on addition of India ink to blood is due to injury to the blood corpuscles, as this fall in antithrombin can be produced also by exposing the blood corpuscles to substances other than India ink.

Unfortunately, attempts to obtain similar findings by addition of toluidine blue or clupein sulphate to heparin plasma as well as to plasma with a maximal antithrombin content from immunization gave only uncertain results in both cases.

Besides the very striking resemblance between heparin and the variable antithrombin component, also other findings appear to point in the same direction.

Thus it is an established fact that heparin is poured out into the blood stream in one of the morbid conditions associated with an increase in antithrombin, namely anaphylactic shock (see Chapter III). This outpouring of heparin into the blood stream proceeds rapidly and is only of brief duration, reminding one therefore strikingly of the primary rise in antithrombin observed in shock. The increase in antithrombin is somewhat more protracted than the rise in heparin, however, but we have to

keep in mind that my experiments are carried out on the rabbit while the heparin studies were carried out on the dog, and that rabbits behave somewhat differently from dogs, especially as to the coagulability of the blood in shock (DYCKERHOFF, MARX & ZIEGLER<sup>40</sup>).

It is to be regretted that my experiment with anaphylactic shock on a dog does not allow any conclusions to be drawn.

In addition, it must be remembered that, while heparin itself is thermostable, heparin + cofactor is thermolabile and shows about the same thermolability as antithrombin.

As to the rapidity of the effect of antithrombin on one hand, and the effect of heparin + cofactor on the other, statements have been made by various authors that seem suggestive of a divergence in this respect, as heparin + cofactor is claimed to have a rapid effect, whereas antithrombin has a slow effect.

If we investigate these features more thoroughly however, we find that these statements are not altogether correct. The effect of a strong heparin plasma solution is rapid, it is true, reaching its maximum in 4-5 min. but the effect of weaker solutions is considerably slower. If heparin is added only in such a dose that the plasma is not quite saturated with regard to its antithrombic capacity, we find that this heparin plasma solution reaches its maximal effect against the thrombin only after about 9-10 min., being thus very reminiscent of the antithrombin, which reaches its maximum in 12-14 min.

The addition of small amounts of heparin to the plasma also makes the experimental conditions more like the natural, as total "saturation" does not take place in nature either—as is evident from the fact that addition of heparin, for instance, to immune plasma is able to give an additional slight rise in the antithrombin content.

This investigation was carried out by incubating the heparin plasma together with the thrombin for varying lengths of time, as described in Chapter IV.

Some characteristic incubation curves are shown in Fig. 14.

From the findings here described it is evident that there are several and most striking points of resemblance between heparin + cofactor and the variable component of the antithrombin.

There are also some features, however, that point away from this result.

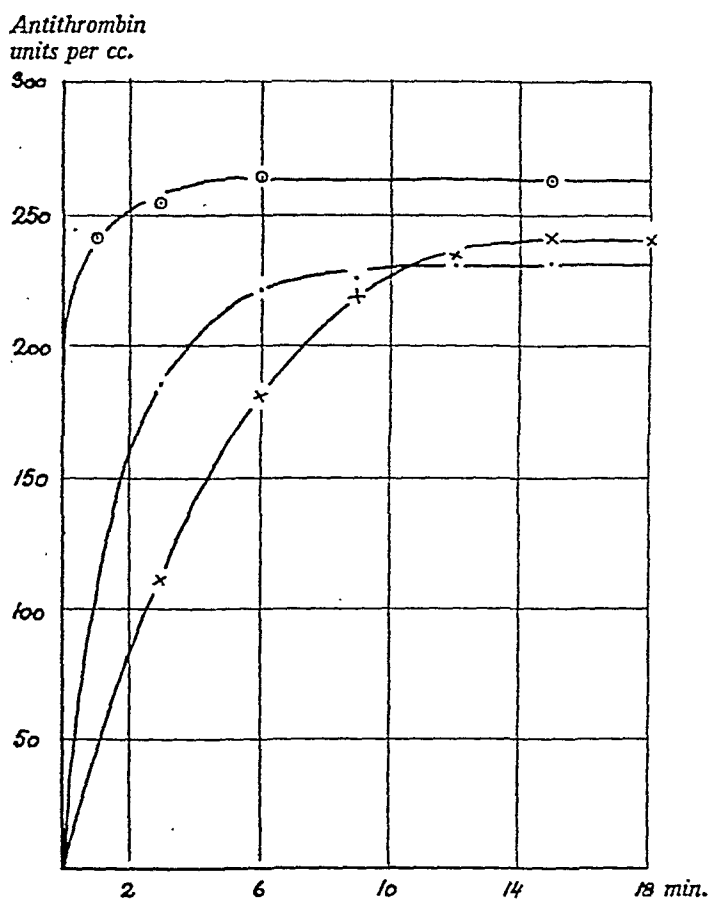


Fig. 14. Curves showing the rapidity of the inactivation of thrombin by the antithrombin in immune plasma and in heparin plasma.

x—x—x = amount of antithrombin in immune plasma.

— — — = — — — — — heparin plasma (8 mg. per cent).

○—○—○ = — — — — — (20 mg. per cent).

As is well known small amounts of heparin give, when added to plasma, a pronounced prolongation of the coagulation time, but even with the maximal antithrombin concentration in the rabbits, I have been unable to demonstrate any change in the coagulability of the blood, although such an antithrombin content should correspond to the presence of 10–12 mg. per cent heparin in the blood.



When this is the case, the variable antithrombin component cannot be due to that heparin we know. But, when there are so many points of resemblance between the variable antithrombin component and heparin + cofactor, *this antithrombin component probably is identical with a combination between the cofactor and a heparin-like substance, perhaps a precursory stage of heparin.*

In this connection, I want to call attention to the circumstance that the heparin preparations we are acquainted with from our experimental work are prepared from the organs by means of extremely crude and radical processes, so that it is hardly fair, perhaps, to expect that these preparations should present quite the same properties as the genuine physiological substance.

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Regarding the question as to the site of the formation of this variable antithrombin component, as mentioned already, the liver appears not to play any particular rôle. On the other hand, the antithrombin reaction observed during immunization, and the possibility of blocking any rise in antithrombin content by means of India ink appear to indicate that the reticulo-endothelial system constitutes the site of the antithrombin formation.

Also the rise in antithrombin content in obstructive jaundice appears to point in the same direction. According to the general view (cf. STARLING<sup>119</sup> and LUNDGAARD<sup>83</sup>), the bile pigments are produced in the reticulo-endothelial system, and this function must be compromised somehow in obstructive jaundice, which may therefore give rise to the stimulation that brings about the antithrombin production.

#### *Recapitulation.*

1. On bleeding after injection of a large amount of India ink, the fall in antithrombin content resulting from the loss of blood is compensated within two days—just as in normal animals. So the India ink is not able to block this rise in antithrombin.
2. After summarizing the various experimental results, the writer arrives at the conclusion that the antithrombin of

the blood consists of two components, one of which is constant while the other is variable.

3. The identity of these two components is discussed, and it is pointed out that the constant component probably not is a real substance but only identical with the enzyme inhibition due to the plasma albumins, while the labile component shows resemblance to heparin + cofactor.
4. The reticulo-endothelial system is pointed out as the probable site for the formation of the variable antithrombin component.

## Chapter X.

### THE SIGNIFICANCE OF ANTITHROMBIN

Various authors (*e. g.*, MELLANBY<sup>84</sup> and QUICK<sup>107</sup>) have emphasized the peculiar fact that plasma and serum are able to inactivate even considerable amounts of thrombin apparently without influencing the coagulation of the blood. As emphasized first by MELLANBY, a certain amount of thrombin clots the plasma and a corresponding solution of fibrinogen in about the same time. MELLANBY and QUICK have tried to explain this phenomenon by assuming that thrombin has far greater affinity for fibrinogen than for antithrombin and hence would be able to combine with antithrombin only when no more fibrinogen was present, or, in other words, not until the coagulation had taken place.

The basis for this theory is entirely speculative, however.

In accordance with the above-mentioned observations, DYCKERHOFF and collaborators<sup>40</sup> have emphasized that in spite of the rise in antithrombin content during immunization and in anaphylactic shock in rabbits and guinea-pigs, it was not possible to demonstrate any prolongation of the coagulation time of the blood.

As mentioned briefly in the preceding chapter, my observations confirm these findings.

In my studies the coagulation time has been determined on fresh citrate plasma. This was recalcified by addition of 0.15 cc. of a 1.5 per cent solution of  $\text{CaCl}_2$  sicc. and then placed quickly in a water-bath of glass at a temperature of  $37^\circ$ . Under steady cautious shaking, the coagulation time was determined as the time that passed before the beginning of coagulation. The coagulation set in rather suddenly and was easy to observe. Two determinations of this kind were made on every specimen of

plasma. The amount of  $\text{CaCl}_2$  added to the plasma was selected so as to be optimal to the given amount of citrated plasma.

The plasma was obtained by centrifuging for 10 minutes (3000 revolutions per min.) of citrated blood taken from an ear vein by means of a Record syringe.

With this technique the coagulation time for the plasma from 20 rabbits was found to be between 45 and 130 seconds.

The method described here is extremely simple, and I cannot see that any of the complicated methods gives so much better results that anything would be gained by their employment.

Such determinations of the coagulation time on plasma before and after immunization and anaphylactic shock were found to give the values recorded in Table 37.

*Table 37. Coagulation Time of Citrated Plasma on Recalcification.*

Rabbit No.	Coagulation time in normal plasma	Coagulation time in immune plasma	Coagulation time in shock plasma
20	1.46 min.	1.20 min.	
35	1.03 -	2.00 -	
36	0.45 -	1.18 -	
37	0.43 -	0.49 -	
40	1.35 -	1.33 -	2.00 min.
41	1.53 -	0.50 -	1.30 -
42	1.33 -	0.45 -	1.27 -

Examination of the coagulation time of the plasma from day to day during immunization gave likewise normal values.

Like MELLANBY and QUICK, I also found a marked disproportion between the antithrombin content of the plasma and its influence on the coagulation.

Thus I found the amount of antithrombin in 1 cc. of serum to be about 150 antithrombin units, which means that 1 cc. of serum is able to neutralize 150 units of thrombin. That this is a large amount is evident from the fact that 150 units of thrombin added to 150 cc. of blood will be able to bring about the coagulation in less than 30 seconds. As the total amount of blood in a rabbit weighing 2500 g. is about 150 cc., it means that 1 cc. of rabbit serum can neutralize an amount of thrombin

sufficient to coagulate the total amount of blood of the rabbit in less than 30 seconds. In spite of this, on recalcification, the rabbit blood coagulates in 1-2 minutes, and the amounts of thrombin which bring about this coagulation must even be very small (cf. ASTRUP & DARLING<sup>10</sup>).

In contrast to the observations reported by DRINKER & DRINKER, my experiments showed that bleeding does not give a larger fall in the antithrombin concentration than was to be expected from the quantity of blood lost. So the reduction in the coagulation time of the blood under these conditions appears not to be connected with the decrease in antithrombin.

Furthermore, as mentioned before, if the antithrombin content is lowered about 20 per cent under the normal level by intravenous injection of 0.5 cc. of India ink per kg., it keeps constant at this level for a long time—over 6 weeks. Four weeks after the injection of India ink, however, the antithrombin content may be raised again by immunization or obstructive jaundice, at any rate above the normal level. This shows that even though the antithrombin content is capable of increasing, it remains at the low level unless some particular stimulation exerts its influence. This appears to indicate that it makes no particular difference to the organism if the antithrombin concentration of the blood is lower than normal.

All the features mentioned here suggest that the significance of the antithrombin to the coagulation is relatively slight.

HOWELL and collaborators assign a great deal of importance to the antithrombin, however, in particular with regard to the stabilization of the circulating blood. They hold that antithrombin inactivates the amounts of thrombin that sometimes may be formed during the circulation of the blood and thus prevent intravascular coagulation. But this theory rests on an entirely speculative basis.

The failure of antithrombin to influence the coagulation time of the blood may possibly be due to the circumstance that the combination of thrombin with antithrombin is a somewhat protracted process, requiring 15 minutes for its quantitative establishment (see Fig. 2). Since the coagulation of plasma takes place as shown in 1-2 minutes, and since the amount of throm-

bin which produces this coagulation must require a certain part of this time for its formation, the time available for any reaction between thrombin and antithrombin must become so very short, that only small amounts of thrombin can become combined with the antithrombin. Still, this explanation is not absolutely adequate, as the spontaneous coagulation involves only small amounts of thrombin.

The reason for the presence of antithrombin in the blood and its apparent lack of influence on the coagulation of the blood still remains an open question.

In conclusion I want to call attention to another point—the very inaccurate methods at our disposal for determination of the coagulation time of mammalian blood. The standard deviation amounts even with the best of the methods to about 100 per cent. Taking into consideration that even the highest measured rise in antithrombin concentration of the blood is only about 70 per cent, it is obvious that a real estimation of the significance of the antithrombin appears impossible until more reliable methods have been worked out for determination of the coagulation time of the blood.

#### *Recapitulation.*

1. Like some previous investigators, the writer points out that in spite of the rise in the antithrombin content it is not possible to demonstrate any prolongation of the coagulation time of the blood, and that there is a striking disproportion between the antithrombin content of the blood, normal as well as abnormal, and the apparent lack of influence of this upon the coagulation.
2. These features are discussed.

## SUMMARY

In the *introduction* some orientating remarks are made about antithrombin itself. It is pointed out that our knowledge of antithrombin is only slight because hitherto it has not been possible to determine its concentration in the blood with any fair degree of accuracy. In 1940-1941, however, ASTRUP & DARLING succeeded in working out a method for quantitative antithrombin determination. This made it possible to take up earlier, often conflicting, findings for revision.

*Chapter I* gives a brief survey of the modern theories concerning coagulation of the blood. They are all based on the schema set up by MORAWITZ in 1904, and the points about which opinions differ are the physicochemical aspects of the components involved and the complicated ways in which they react upon each other. In this survey particular care is taken to show how the various authors assume that the coagulation-inhibiting substances influence the mechanism of coagulation.

*Chapters II and III* give a historical survey of antithrombin, Chapter II dealing with the older investigations, while the more modern views and experimental results are discussed in Chapter III. The border line dividing the more recent from the older literature may be fixed at the year 1918, as at that time a new substance, heparin, was introduced by HOWELL and HOLT into the discussion about the coagulation of the blood. Through further investigation of this substance and its properties new light was thrown on many of the older, often very conflicting, statements and experimental findings.

The literature on the antithrombic activity of heparin is reviewed, and a number of concordant investigations are seen to show that heparin in itself is inactive, and that it is able to inactivate thrombin only when it is combined with an unknown thermolabile cofactor.

The part played by heparin in the thrombin-inactivating properties of plasma and serum is discussed, and it is pointed out that, as only a small amount of heparin is present in the blood under normal conditions while, on the other hand, plasma and serum are able to inactivate considerable amounts of thrombin, only a small part of the normal antithrombin can be due to heparin. This may conceivably be different under pathological conditions. Most investigators think, however, that heparin has nothing to do with the normal antithrombin, and a number of authors claim even that the thrombin-inactivating properties of plasma and serum are due exclusively to the unspecific enzyme inhibiting properties of the plasma albumins. Several authors have found an increase in the antithrombin content of the blood under certain pathological conditions where there is no reason to assume that the amount of the plasma albumins undergoes any particular change. If these findings be correct, the conclusion will naturally be that the above-mentioned simple conception of the antithrombin is erroneous. The aim of the present studies has therefore been primarily to investigate more thoroughly the amount of antithrombin in the blood under these conditions.

In *Chapter IV* a description is given of the measuring technique employed in these studies as elaborated by ASTRUP & DARLING. Its principle consists in adding varying amounts of serum to 1 cc. of a thrombin solution of a given potency and, after incubation of the mixture in a water-bath at 37° for 15 minutes, measuring its activity against a solution of fibrinogen. By comparison with a control solution without serum it is possible to calculate how much thrombin has disappeared and this is taken as equal to the amount of antithrombin originally present. The antithrombin content of the serum is recorded as antithrombin units per cc. serum.

A thorough investigation of the method shows that the standard deviation is less than 5 per cent and that the incubation period of 15 minutes employed is necessary and adequate in every instance. The antithrombin content of the plasma is demonstrated as being exactly the same as that of the corresponding serum. Assays on specimens of blood from the same rabbit and from 80 rabbits show the physiological variation to be about



$\pm 6$  per cent. When the plasma is kept in the ice-box its antithrombin content remains unchanged for more than one week.

*Chapter V* gives first a detailed account of the investigations carried out by BORDET and GENGOU in 1901 and by DYCKERHOFF and collaborators in 1939 on the antithrombin concentration of the blood during immunization. It is pointed out that so far it has merely been realized that this process is associated with a rise in the antithrombin content of the blood, whereas it has not yet been ascertained how, when and to what extent this rise in antithrombin is brought about. According to DYCKERHOFF, the antithrombin formed under these conditions is independent of the presence of thrombin in the protein solutions employed for the immunization; in addition he assumes that he is able to show that this rise in antithrombin involves no species specificity. BORDET & GENGOU had advanced the opposite view, however.

In the studies here presented the immunization was carried out by means of 5 intravenous injections of various protein solutions. This treatment was found to result in a rise in the antithrombin concentration of the blood, and this rise showed strikingly slight variation from one animal to another and from one antigen to another. A maximum was reached in 10–12 days, in nearly every instance after a rise amounting to 60–70 per cent. When the injection of antigen was discontinued after the maximum was reached, the antithrombin concentration fell off slowly, reaching a normal level in 4–6 weeks. When, on the other hand, the injection of antigen was continued, the antithrombin concentration kept at the high level for a considerable length of time, but it was not possible to obtain an additional rise. Besides this slow and protracted rise in antithrombin content from the fifth day following the first injection, each of the subsequent injections was followed immediately by a brief rise in antithrombin that lasted only about 1 hour and amounted only to 10–20 antithrombin units per cc. The fact that no such rise was seen after the first injection, but only after some length of time, appears to indicate that these small antithrombin reactions in immediate connection with the injection of antigen are phenomena of hypersensitiveness.

Like DYCKERHOFF, the writer found the rise in antithrombin to be independent of the presence of thrombin in the solutions employed for the immunization. In addition, the writer found it to be immaterial whether the immunization was carried out with protein solutions or with solutions of high-molecular substances free from antigenic properties, as for instance, starch, gelatine and gum arabic. Injection of these substances resulted in exactly the same antithrombin reaction. With the employment of these substances too, the characteristic brief and small rise in antithrombin was seen to follow each subsequent injection for about 5 days after the first injection. The fact that this antithrombin reaction was not observed after the first injection of the non-antigenic substances mentioned seems to indicate that even though these substances apparently show no antigenic property, their injection is still associated with sensitization of the animal. The hypersensitiveness produced during immunization with proteins as well as on intravenous injection of gelatine, starch and gum arabic is specific.

Intravenous injections of suspensions of corpuscular antigens caused a somewhat different antithrombin reaction: a small rise of short duration in antithrombin content, immediately after the later injections, but no general rise in antithrombin.

In anaphylactic shock produced by means of sensitization with protein solutions and subsequent reinjection of the corresponding protein, the result was first a sharp primary rise in antithrombin. This was only of brief duration, and after an interval of some hours during which the antithrombin concentration stayed at a normal level, there was a slow secondary rise that reached its maximum about 30 hours after the shock. The primary as well as the secondary rise amounted to about 60-70 per cent of the initial antithrombin concentration.

Sensitisation and subsequent reinjection of the corresponding high-molecular substances without antigenic character—starch, gelatine and gum arabic—resulted in exactly the same antithrombin reaction as described under anaphylactic shock elicited with proteins.

Anaphylactic shock produced with suspensions of corpuscular antigens gave only the primary rise in antithrombin concentration.

Thus there is a distinct difference in the effect on the antithrombin formation brought about by solutions and by suspensions.

*Chapter VI* gives a brief account of previous statements concerning the antithrombin concentration of the blood in obstructive jaundice and injury to the liver. From this it is evident that most authors agree to some extent in the view that the antithrombin concentration is increased in obstructive jaundice, whereas opinions differ widely as to the quantitative aspects of antithrombin in other forms of liver injury.

The writer's investigations show that experimental obstructive jaundice is associated with a rise in antithrombin, whereas injury to the liver produced by poisoning with chloroform and with carbon tetrachloride gives a fall in antithrombin, lasting as long as the poisoning. As thus the liver injury in obstructive jaundice was associated with a rise in antithrombin, while in the poisonings it was associated with a fall, the rise and the fall in antithrombin content can hardly be due to the liver injury.

The rise in antithrombin content observed in obstructive jaundice is of a similar magnitude as the rise associated with immunization.

*Chapter VII* deals with the influence of injection of India ink on the antithrombin concentration. It is demonstrated that small injections of 0.1 cc. of India ink per kg. give a brief fall in antithrombin content amounting to about 20 per cent of the initial value, whereas larger injections produce the same fall, which now lasts for several weeks.

After the first two injections, the reaction to repeated injections of 0.1 cc. of India ink per kg. decreases steadily, until finally the injection gives no reaction whatever.

The injection of large amounts of India ink in animals with a maximal antithrombin content of the blood produces a sharp fall to normal level. This holds true irrespective of the way in which the antithrombin content has been raised to maximum.

Besides producing a fall in the antithrombin content, the injection of India ink may also completely block the antithrombin formation above the normal value, as 5 injections of 0.1 cc. of

India ink per kg. prevent any rise in antithrombin during immunization and in anaphylactic shock and obstructive jaundice.

The addition of India ink to citrated blood in vitro gives likewise a fall in the antithrombin content. This influence of India ink on the antithrombin content is shown to be due to the circumstance that the membrane of the blood corpuscles is injured and that the corpuscles are thereby enabled to combine with antithrombin. This effect of India ink is discussed.

In *Chapter VIII* the antithrombin concentration after a considerable loss of blood is investigated fairly thoroughly, and from the experimental findings the resulting fall in antithrombin is seen to follow the dilution of the blood.

In *Chapter IX* attempts were made to remove the antithrombin completely by the combination of injection of India ink and bleeding. This proved impossible, however, as the fall resulting from the bleeding was compensated in two days, notwithstanding the preceding India ink blockade, just as in the case of normal animals.

After recapitulating the various experimental results, the writer arrives at the conclusion that the antithrombin content of the blood probably consists of two independent components.

One of these makes up normally 80 per cent of the antithrombin content of the blood, it is constant, and it is not affected by injection of India ink. Bleeding gives also a fall in the concentration of this component, owing to dilution of the blood, but this fall is compensated again by a rise, irrespective of India ink blockade. The other component makes up normally only 20 per cent of the antithrombin content of the blood, but various pathological conditions may cause it to rise to values many times the initial. By addition of India ink to the blood in vivo as well as in vitro, it is possible entirely to remove this component, and its formation can be blocked completely by injection of India ink.

Experiments carried out to throw additional light on the identity of these two components suggest the possibility that the first component, which normally makes up 80 per cent of the antithrombin, is not a real substance, but that it may be

identical with the inhibiting properties of the plasma albumins as exerted by simple adsorption.

In contrast hereto, the other, variable component shows some striking points of resemblance to heparin + cofactor.

Finally, the reticulo-endothelial system is pointed out as the probable site of formation for the variable antithrombin component.

In *Chapter X* the writer discusses the significance of the normal as well as pathological antithrombin concentrations. On this point no exact information has been gained by the experiments here reported, and the question has to be left so far to entirely theoretical considerations.

## DANISH SUMMARY

Indledningen er formet som en Orientering om selve Antithrombinet. Det paapeges her, at Kendskabet til Antithrombin kun er ringe grundet paa, at det hidtil ikke har været muligt at bestemme dets Mængdeforhold i Blodet blot med nogenlunde Nøjagtighed. Dette blev imidlertid afhjulpet 1940—1941, idet det lykkedes for ASTRUP og DARLING at udarbejde en Metode til en kvantitativ Antithrombinbestemmelse, der tillod Maalinger med en Nøjagtighed paa under 5 %. Herved blev det muligt at tage de tidligere, ofte modstridende Angivelser op til Revision.

I Kapitel I gives en kortfattet Oversigt over de moderne Koagulationsteorier. Disse hviler alle paa det af MORAWITZ i 1904 opstillede Skema, og de Punkter, hvorom der nu er Uenighed, er de implicerede Komponenters fysisk-kemiske Forhold og de komplicerede Maader, hvorpaa de reagerer med hinanden. Der er ved denne Oversigt særlig lagt Vægt paa at vise, hvorledes forskellige Forfattere tænker sig, de koagulationshæmmende Stoffer influerer paa Koagulationsmekanismen.

Kapitel II og III giver en historisk Oversigt over Antithrombinet, saaledes at der i Kapitel II nærmere gøres Rede for de ældre Undersøgelser, medens de mere moderne Anskuelser og Forsøgsresultater diskuteres i Kapitel III. Skillelinien mellem den ældre og den nyere Litteratur trækkes naturligst omkring 1918, idet da et nyt Stof, Heparinet, af HOWELL og HOLT indførtes i Diskussionen om Blodets Koagulation. Ved nærmere Udforskning af dette Stof og dets Egenskaber kastedes der nyt Lys over mange af de ældre ofte yderst modstridende Angivelser og Forsøgsresultater.

Litteraturen over Heparinets Antithrombinvirkning gennemgaas, og det ses herved, at en Række samstemmende Undersøgelser viser, at Heparinet alene er uvirksomt, og først naar

det kobles til en ukendt, termolabil Co-Faktor bliver det i Stand til at inaktivere Thrombin.

Heparinets Andel i det normale Plasma og Serums thrombin-inaktiverende Egenskaber gøres til Genstand for nærmere Diskussion, og det paavises, at da Heparin under normale Forhold kun findes i Blodet i smaa Mængder, medens paa den anden Side Plasma og Serum er i Stand til at inaktivere betydelige Thrombinmængder, kan kun en ringe Del af det normale Antithrombin skyldes Heparin. Dette kan maaske tænkes at ændre sig ved pathologiske Forhold. De fleste Forskere mener dog, at Heparinet intet har at gøre med det normale Antithrombin, og en Række Forskere hævder endog, at Plasma og Serums thrombininaktiverende Egenskaber udelukkende skyldes Plasmaalbuminernes uspecifikke Enzymhæmning. Specielt udfra en Række Angivelser fra forskellig Side over Fremkomsten af Antithrombinstigning ved pathologiske Tilstande, hvor der ikke er Grund til at formode, at der sker Ændringer i Plasmaalbuminernes Mængdeforhold, konkluderes det, at saafremt disse Angivelser er rigtige, er denne simple Opfattelse af Antithrombinet urigtig. Mine Undersøgelser er derfor i første Linie gaaet ud paa at undersøge Antithrombinmængden ved disse Tilstande nøjere.

I Kapitel IV beskrives den anvendte Maaleteknik, der er angivet af ASTRUP og DARLING. Princippet i denne er, at man til 1 cm<sup>3</sup> af en Thrombinopløsning af bestemt Styrke sætter varierende Serum-mængder, og efter at Blandingen er blevet inkuberet i Vandbad ved 37° i 15 Minutter, maales dens Aktivitet over for en Fibrinogenopløsning. Ved Sammenligning med en Kontrolopløsning uden Serum kan det udregnes, hvormeget Thrombin der er forsvundet, og dette sættes lig Mængden af Antithrombin. Antithrombinmængden angives som Antithrombinenheder pr. cm<sup>3</sup> Serum.

Ved nøjere Undersøgelser af Metoden vises det, at dens Maalefejl er paa under 5 %, og at den anvendte Inkubationstid paa 15 Minutter i alle Tilfælde er nødvendig og tilstrækkelig. Det paavises, at Antithrombinmængden i Plasma og Serum er nøjagtig ens. Ved Undersøgelser paa Blodprøver dels fra samme, dels fra 80 forskellige Kaniner ses det, at den fysiologiske Variation er paa  $\pm 6$  %. Antithrombinmængden i Plasma holder

sig uændret i over 8 Dage, naar Plasmaet opbevares i Isskab.

I Kapitel V gøres først nærmere Rede for de af BORDET og GENGOU i 1901 og af DYCKERHOFF og Medarbejdere i 1939 foretagne Undersøgelser over Antithrombinmængden ved Immunisering, og det paavises, at man endnu kun er naaet til at erkende, at der herved sker en Forøgelse af Blodets Antithrombinindhold, men at man endnu er uden Viden om, hvorledes, hvornaar og hvor stor denne Antithrombinstigning er. Ifølge DYCKERHOFF var det dannede Antithrombin uafhængigt af, om der fandtes Thrombin i de til Immuniseringen benyttede Proteinstofopløsninger, og han mente desuden at kunne vise, at det var uden Artspecifitet. BORDET og GENGOU var derimod af den modsatte Opfattelse. Ved mine Undersøgelser foretoges Immuniseringerne ved 5 intravenøse Injectioner af forskellige Proteinstofopløsninger. Det viste sig, at der herved fremkom en Antithrombinstigning, og at denne forløb overordentlig ensartet fra Dyr til Dyr og fra Antigen til Antigen. I Løbet af 10—12 Dage naaedes et Maximum, næsten i alle Tilfælde efter en Stigning paa 60—70 %. Ophørtes med Antigeninjectionerne efter at Maximum var naaet, faldt Antithrombinmængden langsomt til normal Værdi i Løbet af 4—6 Uger. Fortsattes derimod med Antigeninjectionerne holdt Antithrombinmængden sig paa det høje Niveau i længere Tid, men det var ikke muligt at opnaa en yderligere Stigning. Foruden denne langsomme og langvarige Antithrombinstigning fremkom der fra 5. Dag efter 1. Injection ved hver af de følgende Injectioner smaa kortvarige Antithrombinstigninger, der blot var af ca. 1 Times Varighed og kun beløb sig til 10—20 Antithrombinenheder pr.  $\text{cm}^3$ . Det, at dette ikke skete ved den første Injection, men først naar der var gaaet nogen Tid, synes at vise, at disse smaa Antithrombinreaktioner, umiddelbart i Tilslutning til Antigeninjectionerne, er et Overfølsomhedsfænomen.

Det viste sig i Lighed med DYCKERHOFF's Iagttagelser, at Antithrombinstigningen var uafhængig af, om der var Thrombin i de Opløsninger, der benyttedes til Immuniseringen; men foruden dette fandtes ogsaa, at det var ligegyldigt, om der benyttedes Proteinstofopløsninger eller Opløsninger af højmolekylære Stoffer uden Antigenkarakter som for Eks. Stivelse, Gelatine



og Gummi Arabicum, idet der ogsaa ved Injection af disse Stoffer fremkom nøjagtig de samme Antithrombinreaktioner. Saaledes kom der ogsaa her ca. 5 Dage efter første Injection ved hver af de følgende den karakteristiske, kortvarige lille Antithrombinstigning, og det, at dette ogsaa i disse Tilfælde først skete ved de senere Injectioner og ikke ved de første, synes at tyde paa, at der ogsaa ved Injection af saadanne Stoffer, der tilsyneladende er uden Antigenkarakter, alligevel sker en Sensibilisering. Saa vel ved Immunisering med Proteinstoffer som ved intravenøs Injection af Gelatine, Stivelse og Gummi Arabicum er Overfølsomheden specifik. Ved intravenøs Injection af Opslemninger af corpusculære Antigener fremkom en noget anden Antithrombinreaktion, idet der herved kun skete de smaa kortvarige Antithrombinstigninger i Tilslutning til de senere Injectioner, medens den generelle Stigning udeblev.

Ved det anaphylaktiske Shock, fremkaldt ved Sensibilisering med og efterfølgende Reinjection af Proteinstofopløsninger, fremkom først en brat primær Stigning. Denne var kun kortvarig, og efter et Interval paa nogle Timer, hvor Antithrombinet laa paa normal Værdi, kom der derpaa en langsom sekundær Stigning, der naaede Maximum ca. 30 Timer efter Shocket. Saa vel den primære som den sekundære Stigning var paa ca. 60—70 %.

Ved Sensibilisering og efterfølgende Reinjection af højmolekylære Stoffer uden Antigenkarakter som for Eks. Stivelse, Gelatine og Gummi Arabicum fremkom nøjagtig de samme Antithrombinudslag som beskrevet ved det anaphylaktiske Shock fremkaldt ved Proteinstofopløsninger.

Ved anaphylaktisk Shock fremkaldt ved Opslemninger af corpusculære Antigener fremkom kun den primære Stigning.

Der er saaledes en tydelig Forskel paa den Virkning paa Antithrombindannelsen, der foraarsages af Opløsninger og Opslemninger.

I Kapitel VI gives en kortfattet Redegørelse for de tidligere Angivelser over Antithrombinmængden ved Staseicterus og Leverbeskadigelser, og det fremgaar heraf, at der foreligger nogen Overensstemmelse, hvad angaar den Opfattelse, at der sker en Antithrombinstigning ved Staseicterus, medens der her-

sker stor Uenighed om Antithrombinets Mængdeforhold ved andre Leverbeskadigelser.

Ved mine Undersøgelser fandtes ved eksperimentel Staseicterus en Antithrombinstigning, medens Leverbeskadigelser fremkaldt ved Intoxikationer med Kloroform og Tetraklorkulstof, saa længe Intoxikationen varede, gav et Antithrombinfald. Da saaledes Leverbeskadigelse ved Staseicterus gav en Antithrombinstigning, medens den ved Forgiftninger gav et Antithrombinfald, kan saavel Antithrombinstigningen som Antithrombinfaldet næppe tilskrives Leverbeskadigelsen.

Antithrombinstigningen ved Staseicterus var af lignende Størrelsesorden som den ved Immunisering.

I Kapitel VII undersøges Tuschinjectionernes Indflydelse paa Antithrombinet. Det paavises, at smaa Injectioner à  $0,1 \text{ cm}^3$  pr. kg giver et kortvarigt Fald paa ca. 20 %, medens større Injectioner ganske vist kun fremkalder det samme Fald, men at dette nu er af flere Ugers Varighed.

Ved gentagne Injectioner à  $0,1 \text{ cm}^3$  Tusch pr. kg faas efter de 2 første Injectioner stadig mindre og mindre Udslag, indtil der til sidst slet ikke sker nogen Antithrombinreaktion.

Injectioner af store Tuschmængder hos Dyr med Maximum af Antithrombin i Blodet fremkalder et brat Fald helt til normal Værdi. Dette gælder, uanset paa hvilken Maade Antithrombinmængden er bragt op til Maximum.

Foruden at fremkalde et Fald i Antithrombinmængden kan Tuschinjectioner desuden fuldstændig blokere dets Dannelse, idet 5 Injectioner à  $0,1 \text{ cm}^3$  Tusch pr. kg hindrer enhver Antithrombinstigning ved Immunisering, anaphylaktisk Shock og Staseicterus.

Tilsætning af Tusch til Citratblod in vitro giver ligeledes et Fald i Antithrombinmængden, og det paavises, at Tuschs Indvirkning paa Antithrombinet skyldes, at Blodlegemernes Membran beskadiges, og at de derved bliver i Stand til at binde Antithrombinet. Iøvrigt gøres Tuschvirkningen til Genstand for nærmere Diskussion.

Antithrombinmængden ved større Blodtab undersøges nøjere i Kapitel VIII, og det ses af Forsøgsresultaterne, at det fremkomne Antithrombinfald følger Fortyndingen i Blodet.

I Kapitel IX forsøgte ved en Kombination af Aareladning og Tuschinjectioner helt at fjerne Antithrombinet. Dette viste sig imidlertid umuligt, idet det ved Aareladningen fremkomne Fald, trods Tuschblokada, alligevel kompenseredes i Løbet af 2 Dage, ligesom Tilfældet var hos normale Dyr.

Efter en Rekapitulation af de forskellige Forsøgsresultater konkluderes, at Blodets Antithrombin sandsynligvis bestaar af 2 Komponenter.

Den ene af disse udgør normalt 80 % af Blodets Antithrombin-indhold, den er ret konstant og paavirkes ikke af Tuschinjectioner. Ved Aareladning faas ogsaa et Fald af denne Komponent grundet paa Blodets Fortynding, men dette Fald kompenseres igen ved en Stigning, uanset Tuschblokada.

Den anden Komponent udgør normalt kun 20 %, men kan ved forskellige patologiske Tilstande bringes til at stige til det mangedobbelte. Tuschtilsætning til Blodet saavel in vivo som in vitro kan helt fjerne denne Komponent, ligesom det kan lykkes med Tusch fuldstændig at blokere dens Dannelse.

Ved Forsøg paa at faa nærmere Klarhed over Identiteten af disse 2 Komponenter fremsættes den Mulighed, at den første af disse, der normalt udgør 80 %, kan være identisk med Plasma-albuminernes Enzymhæmning ved simpel Adsorption.

Den anden variable Komponent udviser derimod slaaende Lighedspunkter med Heparin + Co-Faktor, hvilket nærmere diskuteres.

Til Slut i dette Kapitel paapeges det reticuloendotheliale System som det sandsynlige Dannelsessted for den variable Antithrombinkomponent.

Betydningen af saavel det normale som det patologiske Antithrombin gøres til Genstand for nærmere Diskussion i Kapitel X. Paa dette Punkt lykkedes det ikke ved mine Forsøg at faa eksakte Oplysninger, og jeg har her maattet nøjes med rent teoretiske Overvejelser.



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ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 5. SUPPLEMENTUM XVI.

FROM THE PHARMACOLOGICAL DEPARTMENT OF THE  
CAROLINE INSTITUTE, STOCKHOLM

QUANTITATIVE STUDIES ON  
ALCOHOL TOLERANCE  
IN MAN

THE INFLUENCE OF ETHYL ALCOHOL ON SENSORY, MOTOR,  
AND PSYCHOLOGICAL FUNCTIONS REFERRED TO  
BLOOD ALCOHOL IN NORMAL AND  
HABITUATED INDIVIDUALS

BY

*LEONARD GOLDBERG*

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*Stockholm 1943*

## Corrections

to *Acta Scandinavica Physiologica*, vol. 5, supplementum XVI:

### Quantitative Studies on Alcohol Tolerance in Man.

On page 7, line 9, read dosage-mortality for ordinary mortality.

› › 25, › 1, › 0.63 for 0.65.

› › › › 2, › (25 subjects) for (25 experiments).

› › › › 5, › ( $\beta \times r \times 60$ ) for ( $\beta \times \gamma \times 60$ )

› › 43, › 9 from foot of page, insert and the blood alcohol curve before  
is obvious.

› › 49, › 8, read mm<sup>2</sup> for mm

› › 54, › 7, › table 12 for table 11.

› › › › 8, › › › › › ›

› › 62, › 1, › disappearance for decrease.

› › › › 16, › appearance for increase.

› › › › 17, › disappearance for decrease.

› › 90, › 21, › 30 % for 40 %.

› › 91, in table 7, col. 13 read -7.3 % for -73.2 % and -29.8 % for -40.8 %.

› › 102, line 16, read 1.41 for 1.35.

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## Preface.

Within the scope of study at this laboratory on the action of ethyl alcohol, work on intoxication and habituation was natural. I wish to express my deep gratitude to Professor GÖRAN LILJE-STRAND, Chief of the Pharmacological Department, for his invaluable help and advice during the years which I have had the privilege of working under his guidance.

I am greatly indebted to colleagues at the Caroline Institute as well as to those in other quarters for all their suggestions and criticism, and also to the staff of the Pharmacological Department for their expert technical assistance.

The expenses have been defrayed by grants from the Foundation »Therese och Johan Anderssons Minne», the »Lindahl Fond» of the Royal Swedish Academy of Sciences, »C. A. Nobels Fond», and from the Swedish Society for Medical Research.

Stockholm, April 1943.

LEONARD GOLDBERG.



## Introduction.

The varying tolerance to alcohol in man is a well-known fact, it being the reaction of an individual to the administration of a drug. In the case of ethyl alcohol it is equivalent to the inverse value of the degree of intoxication. Its presence is easily demonstrated.

On the basis of medico-legal examination the number of individuals, denoted as being 'under the influence', is plotted against the prevailing blood alcohol concentration. The resulting graph (fig. 1) shows the typical *S*-shape of an ordinary mortality curve, *i. e.* the Gaussian probability integral. The varying tolerance to alcohol thus seems to be normally distributed among a population.

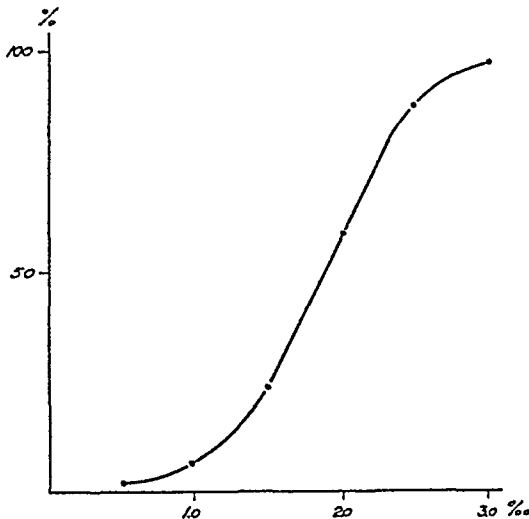


Fig. 1. Frequency of diagnosis "influenced" referred to blood alcohol concentration.

The curve is based on 700 cases, examined by 7 observers, reported by LILJESTRAND (1940).

Two main things may have a bearing upon this:

1. The varying tolerance may be only apparent and the curve a composite one. In that case it would be the result of single examinations made by a number of observers using different



criteria for their diagnoses. This is true on the one hand (LILJE-STRAND 1940), on the other the results of a single investigator give the same principal features. CRAMÉR (1937) has shown that the composition of two probability curves gives another probability distribution, and that the Gaussian function is the only one having this property.

2. Thus the curve must be interpreted as a real expression for a varying tolerance to alcohol, and is probably due to the same biological factors that cause varying tolerance to substances in general, and which combine themselves at random.

Plotting the frequency curve against blood alcohol concentration eliminates variations due to dosage, rate of absorption, of distribution and of elimination. The principal cause of the varying tolerance demonstrated is thus due to variations in *resistance*, which is here defined as the ability of the cells to resist the effect of a drug. The degree of the resistance is generally common to a whole group of individuals as a species, a sex, a stock of animals etc,

So far we can establish that there exists a varying tolerance and resistance to alcohol, the nature of which, however, is still unsolved.

Following the *course* of alcohol intoxication in an individual, and referring the degree of the symptoms to blood alcohol will be the line along which this problem is to be approached.

Comparatively few investigations have been made into the mechanism of the tolerance of an individual and the relationship between symptoms and known physical and chemical properties of the alcohol metabolism, *i. e.* dosage, rate of absorption, of distribution and of elimination or the prevailing blood alcohol concentration. MILES' (1924) studies on the effect of small quantities of alcohol on a series of different functions indicate that a relationship exists between the degree of disturbance and the urine alcohol. GRAF (1933) suggested a proportionality between the square of blood alcohol concentration and the symptoms, which was confirmed by STRAUB (1938).

The purpose of this work is to study quantitatively the degree of disturbance of certain functions in man, to repeat these experiments on differing groups of individuals under different conditions, and finally to evaluate the possible relationship between symptoms and blood alcohol.

Methods were worked out for the recording and measurement of symptoms in two *sensory* functions; viz. the fusion frequency

of the eye to intermittent light, and the sensitivity of the cornea to air stimuli; further in *motor* functions by a test of standing steadiness and by a finger-finger test; and finally in some *psychological* functions by a subtraction and a Bourdon test (part I). The results were treated statistically, and a theory derived from the relationship between the degree of intoxication and blood alcohol, which indicated a direct proportionality between the logarithm of the magnitude of disturbance and the blood alcohol concentration. An approach was also made to the problem of possible differences in intoxication during the rising and the decreasing phase of the blood alcohol curve.

As a consequence of the assumption that there exists a close relationship between symptoms and blood alcohol in one and the same individual, the question arises whether the ingestion of *food* with alcohol, which is known to lower the subsequent blood alcohol level and the degree of intoxication, will alter the relationship or not. The latter possibility seems to favour the assumption that the influence of food on the degree of intoxication is entirely due to its lowering the blood alcohol. This will be the main topic of part II.

A central problem when discussing the varying tolerance to alcohol is that of *habituation*, if such a phenomenon really exists, and its possible bearing upon tolerance or resistance to alcohol.

Most workers in this field agree in assuming an habituation to alcohol, *i. e.* a changed reaction after using alcohol for a long period of time. The habituation generally appears in the form of *increased* tolerance, *i. e.* a lowered degree of intoxication after ingestion of doses that would cause pronounced symptoms in the abstainer.

Opinions differ, however, as to the *causes* of this habituation. Three main conceptions can be distinguished in the discussion.

The first assumes that a certain degree of intoxication always corresponds to one and the same alcohol content of the tissues in all individuals. The habituated individual acquires an ability to rid itself of ingested alcohol more quickly than the unhabituated one, and this enables him to consume large quantities without being affected.

A second conception claims that the habituated individual acquires the ability to compensate psychically the symptoms of intoxication, above all functions which are familiar to him.

A third theory maintains that the process of habituation lies

in a changed reaction of the nerve cells to the penetration of alcohol.

It must not be forgotten in this discussion that habituation may be due to a combination of any two or three of the theories presented.

By referring the degree of disturbance after alcohol ingestion in the functions already described to blood alcohol and by comparing the results for three different groups of individuals: abstainers, moderate drinkers and heavy drinkers, the problem of habituation will be approached in part III. Finally the general problem of tolerance will be discussed on the basis of the evidence brought forward in the previous parts. The influence of variations in absorption, distribution and elimination of alcohol, and of the variations in resistance due to habituation, will be analysed.

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## PART I.

# **The Influence of Alcohol on Sensory, Motor and Psychological Functions in Normal Individuals.**

Investigations on the influence of alcohol on isolated functions have been carried out to a great extent. Comparatively few have, however, dealt with the problem of studying the changes in somatic and psychic functions in one and the same individual, and made records of tests employed for clinical examination and referred to blood alcohol.

DODGE and BENEDICT (1915) investigated the influence of alcohol on the performance of somatic and psychological tests, and reported an impairment of neuro-muscular co-ordination and an increase of the sensory threshold for pain stimulation. Whenever apparent excitation occurred, it was demonstrably or probably due to a relative over-balancing depression of the controlling and inhibiting processes. Their experiments were repeated under somewhat different conditions by MILES (1924) with an improved technique and referred to urine alcohol. He stated that alcohol taken in diluted form gave a well defined and measurable depression in physical and mental processes, which approximately followed the course of the alcohol.

CARLSON *et al.* (1934) in a similar work studying the influence of beer on a series of functions of somatic and psychological nature, failed, however, to show a deviation from normal in other than neuro-muscular functions.

In part I of this paper the influence of alcohol on sensory, motor and psychological functions will be studied quantitatively.

### **.General Experimental Conditions.**

The aim of this work from the methodological point of view is to follow the *course* of alcohol intoxication in man after ingestion of ethyl alcohol. For this purpose a series of tests on different

functions was repeated on one and the same individual at certain intervals before and after alcohol ingestion, and the results were referred to blood alcohol.

The experiments were arranged in the following way:

The subject came on an empty stomach at 8.00 a. m. A short case report was taken and then the experiments began with a *basal* period. During this time the tests, eight in all, were carried out twice, each set of tests lasting about 30 to 40 minutes. The values found served as basal values for the evaluation of the results in the alcohol series (p. 16).

After the basal period alcohol was given. The sets of tests were repeated another seven times at intervals of 40 to 60 minutes. Thus nine complete sets in all were carried out. Some of the tests (both finger-finger and corneal) were repeated twelve to thirteen times at shorter intervals. Each experiment lasted for seven to nine hours, the control period taking about one and a half and the alcohol six to seven hours. Blood alcohol samples were taken, and the result of the tests was referred to the prevailing blood alcohol concentration.

*Fasting and food:* The *fasting* experiments were made after twelve to fourteen hours of fasting. The subjects in the fasting experiments were sometimes allowed to eat a light meal about four after the ingestion of alcohol hours. The absorption was at that time completed and the symptoms had nearly disappeared.

In the *food* experiments the basal period, *i. e.* the tests previous to the alcohol ingestion, was made on an empty stomach. Then the food was taken, and during the last ten minutes the alcohol. In a preliminary series the food consisted only of one or two slices of bread and butter or some cake. In the final experiments a substantial meal was given, consisting of sandwiches,  $\frac{1}{2}$  liter milk, porridge and a warm course of meat and potatoes or fried fish, etc.

*Alcohol dosage:* The alcohol used was ordinary Swedish Potato brandy about 40 vol. %, corresponding to a concentration of alcohol of 31.7 g per 100 cc. It was generally given neat. Sometimes a few cc of vermouth or madeira were added in order to improve the taste, above all to abstainers who showed difficulty in swallowing the whole dose.

The quantity of alcohol administered varied between 120 and 340 cc, corresponding to a dosage of 0.63—1.42 g of alcohol absolute per kg body weight, the whole dose taken within ten minutes.

Blood alcohol samples, double or triple samples, were taken by means of WIDMARK capillaries (WIDMARK 1932) with 20—30 minutes interval during absorption, and with an interval of 40—60 minutes during the post-absorptive period. Nine sets of determinations were made in each experiment.

The blood alcohol analysis was carried out according to the micro method of WIDMARK (1932), and the result expressed in  $\text{‰} = \text{mg alcohol per gram blood}$ .

Calculation of  $\beta$  and  $c_0$  was made according to the method of least squares (WIDMARK 1932) or more easily according to FISHER (1936). In some cases the standard errors of  $\beta$  and  $c_0$  were also calculated (BERNHARD and GOLDBERG 1935). If the blood alcohol samples are taken at regular intervals during the post-absorptive period, the calculations can be considerably simplified.<sup>1</sup>

As the first value for  $\beta$  calculation the determination 120—150 minutes after alcohol ingestion was taken.

*Test subjects:* The subjects belonged to three main groups according to their alcoholic habits and degree of habituation to alcohol. The division in groups was made previous to the alcohol experiments.

*A. Abstainers (A)*, eleven in all. They had never or only once or twice a year taken alcohol. They were mostly students and served for four control experiments and thirteen alcohol ones (part III). The alcohol dosage in this group was 0.63—1.0 g alcohol abs. per kg.

*B. Moderate drinkers (M)*. This group comprised twenty-five subjects, who used alcohol in moderate doses. Their alcoholic habits varied from taking small quantities of alcohol occasionally when offered, to taking it rather regularly up to one to two liters of potato brandy a month. They came from all social classes; of the seventeen who served for the experiments in part I, fourteen were students. With this group forty experiments in all were made, nine preliminary, eight control, and twenty-three with alcohol. The dosage was 1.0—1.42 g alc. abs. per kg.

*C. Heavy drinkers (H)*. These subjects, fourteen in all, were used to taking large quantities of alcohol, *i. e.*  $\frac{1}{4}$ — $\frac{1}{3}$  litre Swedish potato brandy daily or almost daily. Of the ten subjects who served for the experiments of part III, three were highly educated people, and the others workmen of varying education. Three were

<sup>1</sup> The author is indebted to E. BÁRÁNY, M. D., Upsala University, and Civ. eng. G. SCHACKNE, Stockholm, for their suggesting a practical improvement of the original formulae.

alcoholic addicts in the medico-legal sense, being under the supervision of the Swedish Temperance Board. No one complained of any disturbances due to alcohol consumption or had received medical treatment in a hospital. The dosage of alcohol was 1.2—1.41 g alc. abs. per kg body weight.

*Experimental series:* The experiments consisted of two series, a preliminary and a final one.

A. The *preliminary* series (June 1939 to May 1942) was made up of tentative experiments on all three groups during which different methods were tested on their reliability for alcohol experiments. The results of the blood alcohol analyses (16 expts) are included in the discussion on blood alcohol. The results of the tests were not included.

B. The *final* series (June 1942 to March 1943) was carried out according to the principles described, and comprised fifty-seven experiments in all. In nine of these experiments (summer series 1942) slight variations were made before the final technique was adopted. Only one basal value was made in these experiments prior to the alcohol ingestion. Further the distance between glass tube and eye in the corneal test (p. 39) was varied from subject to subject, and finally only one finger-finger trial was made at each determination (p. 56). As these modifications were shown to have no significant bearing upon the results, these experiments were included in the averages.

*Methods:* In the preliminary experiments eleven methods allowing of quantitatively evaluating changes in nineteen different functions were tested. For the final series of experiments the methods were limited to six, giving consistent values and permitting quantitative records of eight different functions:

A. *Sensory functions.*

1. The fusion frequency of the eye to intermittent light (flicker test).
2. The sensitivity of the cornea to air stimuli (corneal test).

B. *Motor functions.*

1. The area of sway while standing = the standing steadiness (quantitative Romberg sign).
  - a. Ordinary Romberg position for 15 sec.
  - b. Modified       "       "       "       "       "
2. The area of dotting in the finger-finger test.

C. *Psychological functions.*

1. Duration of a series of subtractions (subtraction test).
2. Apprehension, attention and concentration (Bourdon test).
  - a. Number of letters correctly marked (B C).
  - b. Total number of letters read (B T).

The composition and duration of each set of tests was:

1. Corneal test .....	2—5	min.
2. Romberg test .....	1—3	»
3. Finger-finger test.....	2—3	»
4. Bourdon test.....	4	»
5. Blood alcohol sampling .....	2—3	»
6. Adaptation in dark room .....	10—15	»
7. Subtraction test .....	1—2	»
8. Flicker Test .....	3—5	»
		<hr/>
		Total 25—40 min.

The Bourdon test was performed in good illumination with no outside disturbance. The subtraction test was made in the dark room to gain time and to avoid disturbance.

When two subjects were examined at the same time, an assistant performed the Romberg, Bourdon and finger-finger tests and the author the corneal, flicker, and subtraction tests as well as the taking of blood samples.

*Control experiments:* In order to investigate the possible influence of *practice* and *fatigue* on the functions tested, a series of *control* experiments, twelve in all, were carried out. These were arranged exactly as the alcohol experiments, with the only exception that no alcohol was taken. They thus comprised nine complete sets of tests, performed during six to eight hours. The results were evaluated exactly as the alcohol experiments (p. 16).

### Mathematical Analysis.

The experiments were arranged in a manner so as to render possible a theoretical analysis and a statistical treatment of the results as well as their applicability for practical purposes.

For that reason the first object was to make use of methods which allowed of quantitatively evaluating the degree of disturbance by recording or measuring the functions tested.



The second object was to apply measuring units which would permit of a comparison between the different tests, both with regard to their suitability to measure changes in functions under normal conditions as well as after ingestion of alcohol. One phase of this problem was taken into consideration by performing all tests on one and the same individual (p. 12), thus eliminating part of the variability due to heterogeneity of the material.

The third object was to establish the possible relationship between the degree of intoxication and the concentration of alcohol in the blood, in a single individual as well as within and between groups of individuals of differing habituation to alcohol.

### Evaluation.

All experiments were evaluated in the same manner. The results were expressed partly in absolute units, partly transformed to logarithms and related to a basal value by forming the logarithmic differences.

*Basal value:* The mean of the two determinations prior to the ingestion of alcohol served as a basal value. Among the advantages of relating a change in a function to a basal value were that differences between individuals as well as those in one and the same individual from day to day were eliminated. Relative units further permit of comparing different tests with one another. In the control experiments the mean of the first two determinations served as basal value.

The logarithmic basal values were shown to be approximately normally distributed by proving that they fitted the Gaussian probability integral. This was made graphically according to BLISS (1938, 1939): the log basal values were arranged in order of magnitude, their frequency in percentage of the total number converted to probability units and plotted against the log basal value. The curve becomes a straight line if the values are normally distributed. — The data were found to agree within the range of error with expectation.

Average logarithmic basal values for a whole *group* of individuals were computed as the mean of the individual logarithmic basal values.

*Logarithmic difference:* All values found were transformed to logarithms, and the difference between the logarithm of a value found after alcohol ingestion and that of the basal value was termed *the logarithmic difference*.

The logarithmic difference was used as an expression of the degree of symptoms after alcohol ingestion. Its anti-logarithm, multiplied by 100, expresses the magnitude of a value found relative to the basal value. In this case the basal value is equal to 100 %, and the value found the percentage of the basal value. An example will illustrate the computation.

Ex. 1. The finger-finger test (p. 56) gave as result of a first determination an area of dotting of 214 mm<sup>2</sup>; a second determination, 20 minutes later, gave 242 mm<sup>2</sup>. The logarithms are 2.3304 and 2.3838, and the logarithmic mean  $\frac{2.3304 + 2.3838}{2} = 2.3571$ , corresponding to 227.6 mm<sup>2</sup>,\* which served as basal value.

A determination, made 57 minutes after the ingestion of alcohol, gave 484 mm<sup>2</sup>. Its logarithm is 2.6848, and the logarithmic difference 2.6848 — 2.3571 = 0.3277. The anti-logarithm of [0.3277] × 100 is 212.7.

The effect of alcohol on the finger-finger test thus increased the area of dotting by log difference 0.3277 = 212.7 % of the basal area. The percentual increase was 112.7 %, as the basal value was equal to 100 %.

The use of the log difference instead of a percentage difference has several advantages, one being that this way of computation may be applied to increasing as well as to decreasing values. An example applied to the Bourdon test will show this.

Ex. 2. Two trials prior to the taking of alcohol gave as result, that the number of letters correctly marked in 4 minutes (p. 69) was 93 and 101 letters respectively. The log basal value was

$$\frac{1.9685 + 2.0043}{2} = 1.9864. \text{ Its anti-logarithm} = 96.9 \text{ letters.}$$

A new trial 113 minutes after the alcohol ingestion gave only 50 letters. The log difference = 1.6990 — 1.9864 = —0.2874. The anti-logarithm of [—0.2874] × 100 = [0.7126 — 1] × 100 = 51.6 %, corresponding to a decrease of 48.4 %.

Multiplying or dividing the absolute values does not change the logarithmic difference, a property common to logarithms and percentages. With respect to *c. g.* the Bourdon test this means that whether the number of letters correctly marked per 4 minutes or per 1 minute is taken as unit, the same logarithmic or percentage difference is obtained.

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\* The arithmetical mean is 228.0 mm<sup>2</sup>, which shows that there is no essential difference between the logarithmic and the arithmetical mean as far as the original values do not differ more than 10 to 20 % from each other; the difference between the arithmetical and the log mean then is only 0.1—0.45 %.

A characteristic feature of the logarithmic difference which distinguishes it from percentage values, is that the inverse function of the absolute values only changes the sign of the logarithmic difference and not its magnitude. This is one of the reasons why logarithms and log differences have been applied in biological work, *e. g.* concerning potency of drugs etc. (BURN 1937). The example with the Bourdon test will serve to illustrate this.

Ex. 3. The inverse value of the number of letters correctly marked in 4 minutes means the time necessary to mark a single letter. Using the same figures as before (ex. 2), the two trials prior to alcohol ingestion, when evaluated as min/letter, gave 0.0430 and 0.0396 minutes per letter respectively. The log basal value is

$$= \frac{\log 0.0430 + \log 0.0396}{2} = \frac{[0.6336 - 2] + [0.5977 - 2]}{2} = [0.6157 - 2].$$

Its antilog = 0.04127 minutes per letter.

The value after ingestion was 0.080 minutes, its log [0.9031 - 2]. The log difference [0.9031 - 2] - [0.6157 - 2] = [0.2874]. Its antilog = 194.8 %, which means an increase of the time per letter of 94.8 %. The inverse value of the absolute figures thus only shifts the sign of the log difference from - to + without changing its numerical value, whereas the percentual value changes from a decrease of 48.4 % to an increase of 94.8 %.

*Logarithmic Variance.* The log variance (log V) or its square root, the log standard deviation (log  $\sigma$ ), was computed from the logarithmic differences according to current formulae (GADDUM 1932, BURN 1937).

$$V = \frac{S(x - \bar{x})^2}{n - 1} \dots \dots \dots (1)$$

where  $x$  = a value found

$\bar{x}$  = the mean

$n$  = the number of terms.

$S(x - \bar{x})^2$  is computed from the formula

$$S(x - \bar{x})^2 = S(x^2) - \frac{[S(x)]^2}{n} \dots \dots \dots (2a)$$

For log differences formula (2a) becomes

$$S(x - \bar{x})^2 = S(\log^2 \text{ diff.}) - \frac{[S(\log \text{ diff.})]^2}{n} \dots (2b)$$

$$\log V = \frac{1}{n - 1} \left( S(\log^2 \text{ diff.}) - \frac{[S(\log \text{ diff.})]^2}{n} \right) \dots (3a)$$

$$\log \sigma = \pm \sqrt{\frac{1}{n - 1} \left( S(\log^2 \text{ diff.}) - \frac{[S(\log \text{ diff.})]^2}{n} \right)} \dots (3b)$$

The conversion of a logarithmic variance to its anti-logarithm gives the standard deviation expressed as percentage of the mean ( $\sigma\%$ ), which implies a greater positive than negative standard deviation. For values of  $\sigma\%$  exceeding 30—40 %, the difference becomes considerable (cf ex. 2 and 3, where the diff. in percentage is due to the same phenomenon). (See table 5, column 2.) On the other hand logarithms always ought to be applied for computing the variance in biological work, when the standard deviation exceeds 10—15 % (GADDUM 1932, BURNS 1937).

COCHRAN (1938) has derived a formula for approximate transformation of the logarithmic variance ( $\log V$ ) or standard deviation ( $\log \sigma$ ) to a percentual standard deviation ( $\sigma\%$ ).

$$\sigma\% = 230.26 \times \sqrt[4]{\log V} \quad . . . . . (4 a)$$

$$\sigma\% = 230.26 \times \log \sigma \quad . . . . . (4 b)$$

This formula gives values which agree satisfactorily when the percentual standard deviation does not exceed 30—40 %.

From a practical point of view the computation of the standard deviation in logarithms or percentage renders the comparison of the variances of different tests possible (table 5).

### Variability.

a) *Of a single determination.* The variation of a single determination ( $\sigma_s$ ) was ascertained from the differences ( $d_1$ ) between the two determinations prior to the ingestion of alcohol. First the log stand. deviation of the differences ( $\sigma_{d_1}$ ) was calculated by means of formula (3 b), and then  $\log \sigma_s$  according to current formulae (DAHLBERG 1940)

$$\sigma_s = \frac{\sigma_{d_1}}{\sqrt{2}} \quad . . . . . (5)$$

b) *During the day.* The variability of a test during a whole day ( $\sigma_d$ ) was ascertained from control experiments (p. 15), which were evaluated as follows: The mean of the first two determinations served as a basal value, and the subsequent 7 values were related to this by being transformed in logarithms and the logarithmic difference ( $\log$  diff.) computed (p. 16).  $\log \sigma_d$  was calculated according to formula (3 b),  $n$  being 8, and  $\sigma_{d\%}$  according to formula (4 b);

c) *From day to day.* The variations in one and the same individual from day to day ( $\sigma_{dd}$ ) were computed from the differences ( $d_2$ ) between the basal values on two different days in one and the same individual according to formulae (3 b) and (5).

d) *Between individuals.* The variability between different individuals ( $\sigma_i$ ) was ascertained from the standard deviation of the basal values ( $v$ ) of a group of normal individuals.

$$\sigma_i = \sqrt{\frac{S(v - \bar{v})^2}{n - 1}} \dots \dots \dots (6 a)$$

$$\sigma_i = \sqrt{\frac{S(v^2) - \frac{[S(v)]^2}{n}}{n - 1}} \dots \dots \dots (6 b)$$

As all these variances, and standard deviations, were logarithms, they were directly comparable to each other both for one and the same test between individuals as well as between different tests in one and the same individual. For comparison the log standard deviation was also transformed in percentage by means of formula (4 b).

e) *After alcohol ingestion.* A preliminary calculation by analysis of covariance proved that the deviations of the values were greater after alcohol ingestion than under normal conditions (pp. 60, 65), and increased with increasing blood alcohol concentration. The logarithmic variance proved to be rather *constant*. This means that the percentage error did not change with the taking of alcohol.

### Average Curves.

#### Control Experiments.

Average curves for a whole group of subjects were obtained by adding the log differences of every determination with their sign, dividing them by the number of individuals and plotting the averages against time (■—■ in figs. 6, 8, 10, 11, 14, 15, 17 and 18).

#### Alcohol Experiments.

a) *Logarithmic values.* The individual logarithmic differences were plotted against time to show the course of intoxication in the

individual. Two examples of individual graphs are given in figs. 5 and 13.

*Smoothed* average log difference curves for a whole group of individuals were obtained as follows: For every individual the logarithmic differences were read from the individual graph at an interval of 20 min., all values per 20 min., per 40 min., etc. being added, the sums divided by the number of individuals, and the averages plotted against time, giving an average curve (● — ● figs. 6, 8, 10, 11, 14, 15, 17 and 18).

That manner of graphical smoothing gives a clear view of the course of intoxication and will not change the principal features of the average curve, *e. g.* will not turn an exponential curve into a straight line, etc.

b) *Absolute values.* Smoothed average absolute curves were computed by adding the average log difference per 20 min. to the average logarithmic basal value (p. 16) and plotting the anti-logarithms = the average absolute value, against time (× — × figs. 6, 8, 10, 11, 14, 15, 17 and 18).

### Relationship between Symptoms and Blood Alcohol.

#### *Average Curves.*

a) *Decreasing part.* In order to establish the possible relationship between the degree of a symptom and the blood alcohol concentration the average log differences were plotted against the average blood alcohol curve. This was done for all functions tested and within the three groups, abstainers, moderate drinkers and heavy drinkers (figs. 20—28).

The following principles were applied:

The relationship was first computed for the *decreasing* part the first value being that 20 min. after the blood alcohol maximum.

The average log differences were fitted by a straight line, the regression line, calculated from the average values by the method of least squares by means of ordinary formulae; the probability (*P*) that the regression lines coincide with regard to means and slope, was established by analysis of covariance (SNEDECOR 1938, BONNIER and TEDIN 1940).

To test the validity of fitting the data of log differences by a straight line SNEDECOR's test (1938) of deviation from linear regression of group values was applied. The deviations from

linear regression  $\frac{S(y - Y)^2}{h - 2}$  were compared with the error within the groups  $\frac{S(y - \bar{y})}{n - h}$ ,

where  $n$  = the total number of variates, and

$h$  = the number of groups.

The result indicated no more than random sampling departure from linear regression ( $P$  being  $> 0.2$ ). The fitted regression lines thus lay well within the range of experimental error, and gave an approximate expression of the relationship between the decrease in the degree of symptoms (expressed as log differences) and the blood alcohol concentration.

The concentration of alcohol which corresponds to the intersection between the regression line and the  $x$ -axis, expresses the blood alcohol level at which the symptoms have disappeared and the function tested returns to normal. This level was termed the *disappearance* threshold for the symptoms.

Values for average thresholds, slopes and probability of coincidence are given in tables 6 and 11.

b) *Increasing part.* Owing to the few log difference values of the increasing part of the curve, — generally only one or two determinations were made during the first hour — the regression lines for that part of the curves were fitted graphically for a normal group.

The point of intersection between the regression line and  $x$ -axis in this case means the blood alcohol level at which the functions tested begin to deviate from normal, *i. e.* to show beginning disorders. This level represents the *appearance* threshold for the symptoms, and the values are given in table 6.

### *Individual Curves.*

Besides being plotted against time, the individual log differences were also referred to the individual blood alcohol curve (*e. g.* figs. 5 B and 13 D). These diagrams, from which the individual regression lines were graphically computed, were drawn in every experiment. The mean of the individual threshold values and their variability is given in table 12.

Figs. 13 C and D also illustrate the difference in relationship between plotting absolute values and log differences against

blood alcohol. In fig. 13 C the relationship between absolute values and blood alcohol is an exponential curve, whereas plotting log differences gives a straight line. The advantage of using logarithms for graphic computation of threshold (p. 21) and slope is evident.

## The Blood Alcohol Curve in Fasting Individuals.

The principles for the occurrence of alcohol in the body after administration were introduced by GRÉHANT (1895) and NICLOUX (1900), who assumed its distribution to follow the laws of diffusion. This has been quantitatively proved as regards most body fluids: *urine*: WIDMARK (1916), *milk*: OLOW (1923), *cerebro-spinal fluid*: ABRAMSON and LINDE (1930), MEHRTENS and NEWMAN (1933), GOLDBERG (1937 a, b), *saliva*: LINDE (1932), *sweat*: NYMAN and PALMLÖV (1936), *aqueous humour* (BERGGREN, unpublished). Finally absorption of alcohol from the stomach in cat and man was shown to be a pure diffusion process (BERGGREN and GOLDBERG 1940).

The magnitude of the distribution ratio of alcohol for the various tissues after completed absorption depends on their content of fat (CARPENTER 1929) and water (NICLOUX 1934, NICLOUX and GOSSELIN 1934).

The disappearance of alcohol from the body is due to combustion in the liver to 90—97 % (BATELLI and STERN 1910, HIRSCH 1916, FIESSINGER *et al.* 1936, LUNDGAARD 1937, 1938 a, b), and only to 3—10 % (ATWATER and BENEDICT 1902) to elimination by urine, (WIDMARK 1916), by expired air (LILJESTRAND and LINDE 1930), and by sweat (NYMAN and PALMLÖV 1936).

The course of the blood alcohol curve during the post-absorptive period was shown to be rectilinear (MELLANBY 1919, WIDMARK 1930); and WIDMARK introduced the term  $\beta$  for the rate of disappearance of alcohol from the blood, and  $r$  for the distribution ratio between the body and the blood alcohol concentration.

TEORELL (1937 a, b) derived formulae for the calculation of the resulting concentration in the various tissues of a drug after administration, assuming distribution to be a diffusion process.



The apparent rectilinear course of the blood alcohol curve might be explained as the resultant of a series of exponential processes: diffusion, elimination etc.

HAGGARD and GREENBERG (1934) claimed absorption in dogs to last from 3—6 hours, and the blood alcohol curve to be exponential for that reason. Their results could not be confirmed by administering the alcohol intravenously in man (FLEMING and STOTZ 1936) and in dogs (NEWMAN and CUTTING 1936 a).

FIESSINGER *et al.* (1936) and LUNDSGAARD (1937, 1938 a, b) in experiments on liver perfusion clearly proved alcohol to be metabolized in the liver at a constant rate; the combustion in the resting or working muscle was negligible (NYMAN and PALMLÖV 1934, LUNDSGAARD 1937, 1938 a, b). BERNHARD and GOLDBERG (1935) by the method of least squares proved the rectilinear curve to have the least error. Moreover WIDMARK (1932) and NEYMARK and WIDMARK (1936 b) showed the factor  $\beta$  to be constant, when the administration of alcohol was repeated at regular intervals, which discredits an exponential curve. Thus the apparent rectilinear course of the blood alcohol curve is most likely the expression for the constant metabolic rate of alcohol in the liver.

LÜTH (1939) proved  $\beta$  to vary less among monozygotic than among dizygotic twins, indicating its connection with genetic factors. SCHÖNHEYDER *et al.* (1942) proved  $\beta$  to vary more from day to day in one and the same individual than the experimental error. For practical reasons the conception of the rectilinear course of the blood alcohol curve has proved useful and was accepted by a great number of observers for the blood alcohol curve in normal individuals: GRAF and FLAKE (1933), JUNG-MICHEL (1933), FLEMING and STOTZ (1935, 1936), SCHMIDT (1937), ELBEL (1937), NEWMAN and LEHMAN (1937), VASILIU, POPOVICI and ROZARIU (1939).

### Experiments.

The general course of the blood alcohol curves in this material coincided completely with that reported by other workers (p. 25): a rising part, with a more or less marked maximum, sometimes amounting to an absorption peak (BERNHARD and GOLDBERG 1935), the falling part, first at a higher rate, corresponding to the distribution phase, and then at a constant rate, corresponding to the post-absorptive combustion phase.

The dosage varied between 0.65 and 1.42 g per kg.

$\beta$  was  $0.00223 \text{ ‰} \pm 0.00006$  (25 experiments),  $\sigma$  being  $0.000306 \text{ ‰}$ .

$r$  was  $0.714 \pm 0.0112$ ,  $\sigma$  being 0.056.

The combustion rate ( $\beta \times \gamma \times 60$ ) was 96.6 mg alc/kg/hour  $\pm 3.34$ ,  $\sigma$  being 16.7 mg.

The blood alcohol maximum occurred after 86.0 min.  $\pm 5.25$  (32 determinations);  $\sigma$  was 29.3 min.

These figures coincide completely with those previously published for normal individuals, provided the alcohol was taken on an empty stomach (WIDMARK 1932, BERNHARD and GOLDBERG 1935, SCHMIDT 1937).

*Vomiting.* Vomiting may modify the blood alcohol curve in several ways, depending on its occurrence in relation to absorption. If it comes on *during* absorption, previous to the maximum, it is said to be preceded by a standstill in absorption and followed

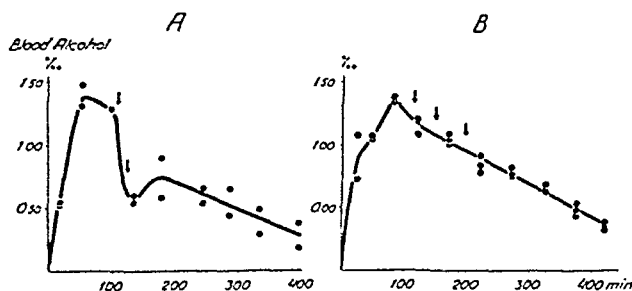


Fig. 2. The influence of vomiting on the blood alcohol.

— Blood alcohol.

↓ Vomiting.

by an increased rate (ELBEL 1937). The findings, illustrated in fig. 2 A, might be interpreted as follows: the vomiting caused a standstill in absorption, and an evacuation of part of the ingested alcohol. Then absorption continued again, causing the slight increase after the last vomiting; after completed absorption the curve turned into the rectilinear falling course.

In the case illustrated in fig. 2 B, the absorption must have been nearly completed, as the vomiting, occurring 124 minutes after ingestion and 32 minutes after the absorption peak; did not alter the rectilinear course of the post-absorptive part of the blood alcohol curve to any noteworthy degree. — This experiment

clearly contradicts the conception of HAGGARD and GREENBERG (1934, 1941) that absorption lasts for 3—6 hours, and that the blood alcohol curve is exponential.

Table 1.

*The Influence of Vomiting on the Blood Alcohol Curve.*

Case	Vomiting in Relation to Blood Alcohol Maximum	Rate of Disappearance $\beta$	Distribution Ratio ( $r$ )
I	During maximum . . . . .	0.00177	0.948
II	After , . . . . .	0.00263	1.276
III	, , . . . . .	0.00218	1.148
IV	, , . . . . .	0.00193	0.950
V	, , . . . . .	0.00173	0.955
Mean		0.00205	1.055

Any influence of vomiting on the metabolic rate was not found, as compared with  $\beta$  of the normal material (table 1). The distribution ratio  $r$  became higher, due to part of the alcohol administered being evacuated by the vomiting and thus never being absorbed (Cf. food p. 88).

## A. The Effect of Alcohol on Sensory Functions.

### 1. The Fusion Frequency of the Eye to Intermittent Light.

The retina has aroused much interest in studies concerning the functions of the central nervous system, as the retinal synaptic layers work as other synapses, their activity being accessible to quantitative measurements also in man.

Investigations have been made on the effect of alcohol on visual acuity, perimetry and dark adaptation. The visual acuity decreased after taking alcohol (MILES 1924, POWELL 1938). MOELLER and BECKER (1939) found a slowing of dark adaptation at concentrations of 0.97 to 2.36 ‰ alcohol in the blood, while no changes were noted in visual acuity nor in visual fields. COLSON (1940) was not able to prove any appreciable changes in visual acuity, visual fields, colour vision nor dark adaptation.

Extensive experiments by GRANIT *et al.* (1930, 1934, 1936), HECHT *et al.* (1934 a, b) and CROZIER *et al.* (1937—41) on the fusion frequency of the eye to intermittent light, have shown this function to be suited for quantitative evaluation. RUBINSTEIN and THERMAN (1935) made some experiments which indicated the fusion frequency to be influenced by alcohol [pers. comm. to GRANIT, quoted from BERNHARD and SKOGLUND (1941)]. Recently BERNHARD and SKOGLUND (1941) showed the fusion frequency in man to decrease by 10—12 % after ingestion of 100—150 cc of 40 % alcohol. On the basis of their experiments the flicker method was assumed to be suitable for quantitative measure of the alcohol effect on the activity of a higher nervous centre, above all of the interaction in the retina. It was primarily decided to use a constant flicker frequency and to ascertain the brightness of light necessary for fusion, which is the principle of the flicker method as used in photometry.

## Experiments.

## Technique.

The apparatus used was based on the principles given by GRANIT and HARPER (1930) for the determination of the fusion frequency of the eye to intermittent light. The device is seen on fig. 3.\*

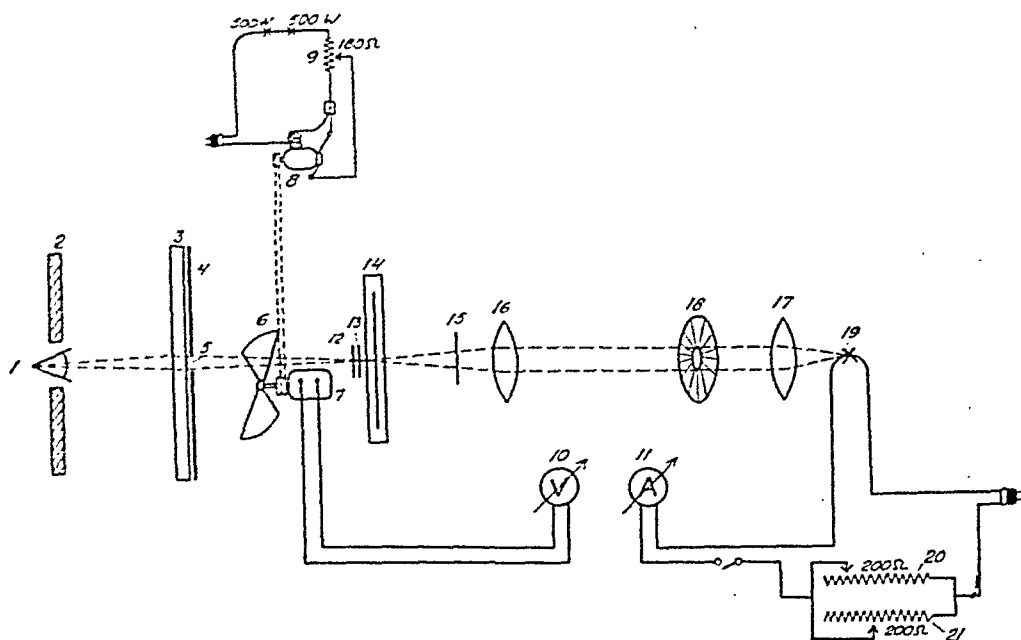


Fig. 3. Device for determination of fusion frequency of the eye.

(Explanation see text.)

A 100 W Kino lamp of 220 V D. C. (19) was used as a light source, focused by means of two lenses (16, 17) onto a neutral wedge (14), and then projected onto a ground glass (3), which was covered by a cardboard (4) with a circular hole (5) in it, 1.75 cm in diameter. The brightness of the light was kept constant by means of two variable rheostats coupled in parallel (20, 21) and controlled by an Ammeter (11) and a Luxmeter before and after each experiment. The intensity could be varied by means of a series of Wratten neutral filters (12, 13) differing 0.10 in density from each other, together with a variable neutral wedge (14) for fine adjustment, covering a range from 0.00—0.25, and allowing

\* The author is indebted to the physicist at the Neurophysiological Laboratory of the Caroline Institute, Mr T. HELME, for valuable help with the apparatus.

a reading of densities of  $\pm 0.0017$ . The wedge was protected from heat radiation by a Uropunctal glass [Zeiss 40] (15). It was calibrated by measuring the transmitted illumination at 7 points along its length by means of a Luxmeter, each point being the mean of 6 determinations. The relation between log intensity transmitted and distance along the wedge was found to be linear (fig. 4 B),

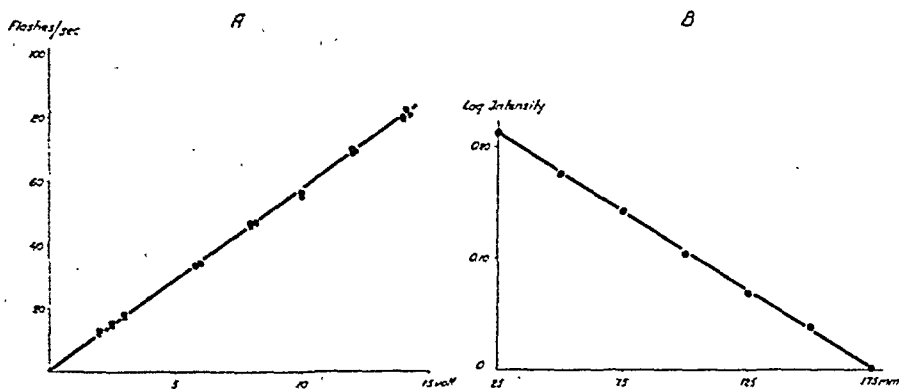


Fig. 4. Calibration curves.

A. Voltmeter: Volt to flashes/sec.

B. Neutral wedge: mm of scale to log intensity.

The flicker was produced by a disc (6) with two symmetrical opaque sectors of  $90^\circ$  each rotating in the beam and directly attached to the axis of a Weston electric tachometer. The tachometer was connected with a Voltmeter (10) calibrated against the alternating current of the mains recorded by a cathode ray oscillograph. The relation between cycles per sec. resp. flashes per sec. and the voltage generated was linear (fig. 4 A). The Voltmeter permitted a reading of  $\pm 0.03$  V, corresponding to  $\pm 0.02$  flashes per sec. The disc was rotated by means of a Singer sewing machine motor (8) coupled according to fig. 3, the rate of which was kept constant within  $\pm 0.1$  cycles per sec. Its speed was varied by means of a variable rheostat (9).

The subject (1) sat in front of the glass screen with his head in a firm head- and chin-rest (2), viewing the light beam binocularly at a distance of 50 cm, thus seeing the diaphragm under a visual angle of  $2^\circ$ .

In order to maintain a constant state of adaptation the experiment took place in a dark room, lit by a 40 W lamp placed

behind and over the subject. The subject was left in the dark room facing the screen for 15 to 20 minutes before each experiment.

The brightness of the background and of the area illuminated, as well as the time of the exposure were kept constant throughout all experiments, each exposure being determined by a Compure shutter set at 1 sec. Only central fixation was used, the subject viewing a cross, drawn with a pencil in the centre of the light patch.

The flicker frequency was generally kept constant at the rate of forty flashes per sec, and the intensity was varied in order to produce fusion. In some experiments the intensity of the light was kept constant and the critical flicker frequency was determined.

Each final value was based on fifteen to twenty readings and was the arithmetical mean of the final six readings made over and under the brightness necessary for fusion or the critical frequency for a given brightness, dependent upon the method chosen.

*Evaluation:* In the experiments with flicker of constant frequency and variable brightness, the intensity was expressed as the negative logarithm of the sum of the filters at the point of fusion, this being the sum of the Wratten neutral filter and the setting on the wedge. — In the experiments with constant intensity and variable flicker frequency, the frequency was expressed in flashes per sec., read from the calibrated tachometer.

## Results.

### Basal values.

It was important to maintain standard conditions in order to obtain consistent values, as variations in area, time of exposure and state of adaptation, involve changes in the functions to be studied, *i. e.* the fusion frequency and the intensity (CROZIER, GRANIT, HECHT, *a. o.*). The conditions used were: 1 sec. of exposure, central fixation, a visual angle of  $2^\circ$ , and a state of constant adaptation, as advised by GRANIT (1936). The binocular fixation involves a slighter scatter of the observations than the monocular (CROZIER and WOLF 1941), the difference being 1 :  $\frac{1}{2}$ .

The flicker method requires co-operation on the part of the

subject as it is a discrimination test. The subject must understand what he has to do and must not change his criterion of fusion or flicker during the experiment. With 2 subjects out of 37 no consistent values were obtained, probably due to this circumstance (cf. p. 33).

It was observed that a lower brightness was needed for fusion when passing from flicker to fusion, than when passing from fusion to flicker, probably corresponding to changes in the two thresholds. The same has been observed by CROZIER and WOLF (1941). A mean value was arrived at by taking the arithmetical mean of the six to eight final readings on each side of the threshold.

*Brightness for fusion:* In 29 normal subjects the log brightness for fusion at a constant flicker frequency of 40 flashes per sec was determined under normal conditions (p. 12) and found to be  $2.681 \pm 0.0777$ .

The *variability* under varying conditions (table 5) was ascertained according to the principles already described (Math. analys. p. 19).

The log variation of a single determination ( $\sigma_s$ ) was 0.0355, corresponding to a coefficient of variation of 8.82 % (23 double determinations on 23 subj.)

The variation during the day ( $\sigma_d$ ) was calculated from 12 control experiments (p. 15) on 12 subjects, where the test was repeated 9 times in 6—7 hours. The log variation was 0.0569, and the coefficient of variation 13.10 %. The slight increase in variation as compared with ( $\sigma_s$ ) will be due to the influence of fatigue. No significant changes in the log brightness for fusion, due to practice of fatigue, were seen during the day (fig. 6 ■—■).

The variation from day to day ( $\sigma_{dd}$ ) in one and the same individual was ascertained in 30 experiments on 13 subjects, each subject serving for at least two experiments on different days; the variation was calculated from the difference in basal value between the two days. If a person had carried out several experiments, the mean of the differences was used for the computation. — The log variation was 0.0768, corresponding to a coefficient of variation of 17.68 %.

The basal values were proved graphically (BLISS 1938, 1939) to be approximately normally distributed, and the log variation between individuals ( $\sigma_i$ ) was 0.4184. The transformation of the logarithmic stand. variation to a coefficient of variation



according to COCHRAN (1938) cannot be performed for reasons already stated (p. 19). In this case the transformation must be made into anti-logarithms. The coefficient of variation was  $\div 162.1\%$  for basal values higher than the mean and  $-71.8\%$  for values lower than the mean.

*Critical flicker frequency:* When using the flicker frequency as a variable factor the variations in the speed of the motor sometimes gave the subject a hint as to how to make his readings. An intelligent person soon learned that fusion occurred when the speed was increased and flicker when it was lowered. The correlation between brightness and flicker frequency on the other hand was very seldom detected. Moreover, the intensity permitted accurate reading of densities of the order of  $\pm 0.002$ , whereas the flicker only permitted a reading of 0.02 flashes per sec. — Out of reasons to be stated (p. 37) only a few experiments, on 6 individuals in all, were performed to determine the critical flicker frequency. The variability during the day, determined in control experiments on two subjects, was  $\pm 0.227$  and  $\pm 0.370$  flashes per sec.

*Relation between critical flicker frequency and intensity.* FERRY (1892) and PORTER (1898, 1902) found the relation between fusion frequency and log intensity to be approximately linear. The frequency rose with increasing intensity. For lower intensities the curves shift to a more horizontal course, as also at very high intensities.

The Ferry-Porter law for the relationship between critical fusion frequency and log intensity thus states that

$$F = a + b \log I \quad . . . . . (7)$$

where  $F$  = flash frequency per time unit

$I$  = intensity of light

$a$  = constant (the ordinata at origo)

$b$  = regression coefficient (the slope of the line).

The Ferry-Porter law is sufficiently accurate for most practical purposes as found, for instance, by GRANIT and HARPER (1930). These observers confirmed the validity of the Ferry-Porter law between 25 and 50 flashes per sec. *i. e.* the linear relationship for flash frequency and log intensity, with varying area and central and peripheral fixation. Beyond that range their curves too tended to follow a more horizontal course.

HECHT *et al.* (1934 a, b) developed a formula, the stationary state equation, which fitted the data of flicker frequency in man for the whole range tested. CROZIER (1937 a) showed that these data could as well be fitted by a logistic or a probability curve. In the experiments to be described below the flicker frequency for the standard intensity varied maximally between 28 and 40 flashes per sec due to the effect of alcohol as well as casual variations. The Ferry-Porter law was therefore applied to these data in order to express the relationship between flash frequency and intensity.

The constant  $b$  was determined in 29 experiments under normal conditions and was 10.0. This value agrees well with those previously given by other observers on normal subjects. From the curves of GRANIT and HARPER (1930) with central fixation, the values of 10.5 to 14.5 can be read off. RUBINSTEIN and THERMAN (1935) found a value of 10.9, and CROZIER *et al.* (1937 c) values between 10.4 and 12.0.

### Alcohol Experiments.

The taking of alcohol implied an increased strain on the subject in the discrimination between fusion and flicker. In most cases the experiments could be completed with only few exceptions, 5 experiments having to be discarded, because no consistent values could be obtained. This occurred more frequently after alcohol than under normal conditions (p. 31). One of the reasons for this is that already described, viz. the inability of some subjects to maintain constant criteria for their discrimination. This inability was accentuated after alcohol ingestion.

Another reason is the presence of an alcohol nystagmus, which generally appeared at concentrations of alcohol in the blood of over 0.7 to 0.8 ‰ (unpublished) and usually only in peripheral fixation. At higher blood alcohol concentrations or in especially sensitive subjects the nystagmus also appeared in central fixation. This often made the fixation of the centre of the illuminated area impossible. The nystagmus was in certain cases so intense that the flicker test elicited vomiting.

Another observation supports the assumption that the alcohol nystagmus may be of significance. A subject with a pronounced nystagmus even under normal conditions could well pass the fusion frequency test. Some time after alcohol ingestion at a blood

alcohol concentration of 1 ‰, when a very frequent alcohol nystagmus was added to his normal nystagmus, he failed to cooperate.

*Brightness for fusion:* When the flicker frequency was kept constant the intensity needed for fusion increased with increasing blood alcohol and decreased with the lowering of the blood alcohol level in the post-absorptive phase. Generally this symptom returned

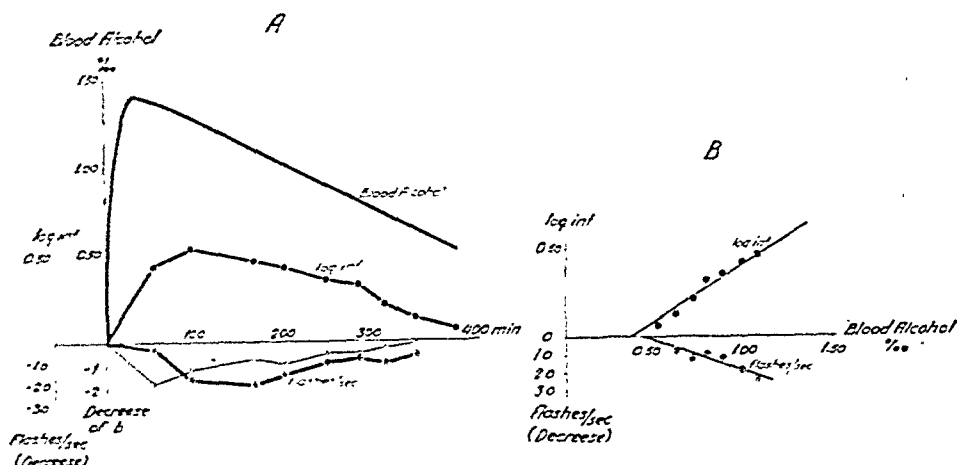


Fig. 5. The flicker test.

Variations in brightness for fusion and fusion frequency in alcohol intoxication.

5. 2. 43. Exp. 8 A. 1.0 g alcohol per kg.

A. Symptoms plotted against time.

B. Symptoms plotted against blood alcohol

- Blood alcohol.
- — ● Brightness for fusion.
- — ○ Fusion frequency.
- — ○  $b = \text{Flashes per sec/log intensity}$ .

to normal before the blood alcohol reached zero level. A typical experiment is illustrated in fig. 5 A, and an average curve, being the mean of 16 experiments on 16 subjects, is given in fig. 6.

When log brightness was referred to the prevailing blood alcohol concentration, an approximate straight relationship was found. This is illustrated in fig. 5 B (● — ●) and in fig. 21 (● — ●) for the decreasing part of the symptoms.

This relationship implies that there seems to exist a threshold value for the symptoms. The appearance threshold means that blood alcohol concentration, at which symptoms begin to appear. The disappearance threshold means that concentration of alcohol

in the blood at which the symptoms disappear and return to normal. The appearance threshold was computed graphically (p. 22) and was 0.54 ‰, the disappearance threshold was calculated from the regression line (p. 22) and was 0.53 ‰ (table 6, cols. 1—3).

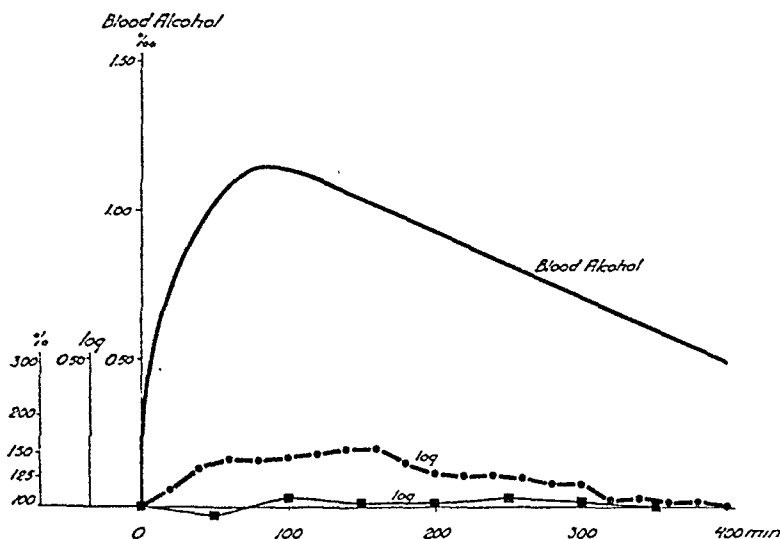


Fig. 6. Brightness for fusion.

Changes during alcohol intoxication. Mean of 14 experiments.

- Blood alcohol curve.
- — ■ — ■ Deviations in control experiments (Mean of 12 experiments).
- — ● — ● Change after alcohol ingestion.

The slopes of the regression lines for the increasing and decreasing symptoms practically coincided; only the slope of the decreasing regression line was calculated (p. 21) and was 0.3596 (table 6, col. 5). By means of the regression line the degree of intoxication at a given level can be calculated, at 1 ‰ alcohol in the blood for instance, it was 151 % (table 6, col. 7) of the normal, that was termed 100 % (p. 17).

*Critical flicker frequency:* In some experiments, nine in all, the flicker frequency was used as the variable factor and the intensity of the light was kept constant. A typical experiment is illustrated in fig 5.

In fig. 5 A, the log intensity and the flash frequency per sec. were plotted against time. The flash frequency decreased with increasing blood alcohol, it reached its minimum somewhat later than the blood alcohol maximum, and then increased with the fall, to reach its original value before the blood alcohol had returned to normal.

In this experiment the constant  $b$  (p. 32), *i. e.* the relation between flash frequency and log intensity, was also determined. The value of the constant varied in the same direction as the flash frequency, *i. e.* in an opposite direction to the blood alcohol. As only two values for the brightness were used to determine  $b$ , the changes were not conclusive.

The two tests, *i. e.* brightness necessary for fusion and critical frequency for a constant brightness, are seen to differ in sensitivity and hence in usefulness as criteria in our experiments. This difference is brought out by the following analysis: The variability of the test in control experiments (nine determinations) for the subject of fig. 5 was for log brightness  $\pm 0.0270$  in terms of log units, and for the critical flicker frequency  $\pm 0.227$  flashes per sec.

In fig. 5 the two functions tested are drawn approximately to the same scale. Log int. 0.10 corresponded to 3.7  $\sigma$  units ( $\sigma = 0.027$ ) in the log intensity scale and 1 flash unit to 4.4  $\sigma$  units ( $\sigma = 0.227$ ). This fact is still more emphasized by the accuracy with which brightness resp. flicker frequencies could be read off from the instruments, as pointed out above (p. 28).

In fig. 5 A the brightness for fusion ( $\bullet - \bullet$ ) showed a maximal increase of 0.53 in log units, corresponding to a degree of intoxication of 339 % (the normal being termed 100 %) at a blood alcohol concentration of 1.27 ‰. The critical flash frequency only diminished by 2.4 flashes per sec., from 32.6 to 30.2. If the flash frequency be transformed to log units, the decrease would correspond to a degree of intoxication of 108 %.

The difference in sensitivity between the two test methods can be demonstrated in another way, *viz.* from the relationship between symptoms and blood alcohol (fig. 5 B). The scales along the  $y$ -axis were the same as in fig. 5 A. The  $x$ -axis in B means blood alcohol concentration in ‰.

Three facts are seen from the graph in fig. 5 B.

1. The disappearance thresholds were about the same.
2. The sensitivity to changes in alcohol concentration of the brightness for fusion is higher than that of the critical flicker frequency, the slope of the intensity curve being steeper, and the scales comparable.
3. The variability of the intensity function under alcohol influence is smaller than that of the flash frequency, the deviations from the regression line being smaller.

The same principal findings as described here were confirmed in eight experiments on other subjects.

For routine work where only one of the functions can be tested it is no doubt more suitable to use brightness for fusion as a variable at a constant flicker frequency, which method was also adopted for the whole series.

The electro-physiological basis for the findings stated is elucidated by experiments on frog reported by BERNHARD and SKOGLUND (1941). These authors studied the effect of alcohol on the electrical response of the frog's eye to light, and stated that alcohol had a selectively suppressing effect on the isolated negative component P III of the electro-retinogram as well as on inhibition in the optic nerve. The statements were confirmed on the pure cone eye of the tortoise (*Testudo*), the retinogram of which is dominated by the negative component P III (BERNHARD 1942).

From the sensory point of view alcohol acts very much like dark adaptation, and BERNHARD and SKOGLUND were able to prove fusion frequency to intermittent light in the frog and in man (p. 27) to be reduced by alcohol, much as it was reduced by dark adaptation in the frog (GRANIT and RIDDELL 1934).

In analogy with the effect of alcohol are investigations on the influence of low oxygen tensions. SEITZ (quoted from MCFARLAND and HALPERIN 1940) observed at 10.6 per cent  $O_2$  a decrease in critical fusion frequency for intermittent light. On the other hand RUBINSTEIN and THERMAN (1935) found hyperventilation to lead to increased critical frequencies for flicker, followed by a drop below the normal upon cessation of hyperventilation.

Similar findings were also reported for other functions of the eye. MCFARLAND and EVANS (1939) reported a rise of the threshold for dark adaptation after inhaling 11.7—15.8 per cent oxygen, and a similar change in visual acuity (MCFARLAND and HALPERIN 1940). Inhalation of air restored normal visual acuity, and 100 per cent oxygen produced a slight improvement. Finally, MCFARLAND and FORBES (1941) stated that hypoglycemia induced the same changes in dark adaptation as did anoxia.

To summarize, the effect of alcohol on the flicker test was shown to follow quantitatively the blood alcohol concentration and con-

sisted of an increase in the brightness necessary for fusion at a constant flicker frequency, and a decrease in the critical fusion frequency at a constant brightness. This is most probably to be interpreted as a suppressing effect of alcohol on the component P III of the electroretinogram.

## 2. The Sensitivity of the Cornea to Air Stimuli.

It is a well-known fact that alcohol depresses the sensitivity to touch and pain in man.

MCDUGALL and SMITH (1920) found an impairment in the ability to discriminate *tactile* impressions on the skin by testing the ability to localize two points 2.5 cm. apart: after taking alcohol the percentage of errors rose from 24 to 90.

DODGE and BENEDICT (1915) used faradisation of the fingertips to produce a sensory excitation, increasing the strength of the current until the test subject felt *pain*, when he had to press a key. They state that the threshold for electrical stimulation is raised after moderate doses of alcohol. With a method similar to that of DODGE and BENEDICT, MILES (1924), on the other hand, observed no conclusive changes in the threshold for pain in two subjects.

CARLSON *et al.* (1934) used the Frey Reizhaar-technique for ascertaining pain sensitivity. They too saw no consistent change in the threshold after small quantities of alcohol, corresponding to a mean blood alcohol concentration of 0.60 ‰. MULLIN and LUCKHARDT (1934), however, by employing the same technique found a distinct decrease in pain sensitivity lasting about two to three hours after the administration of 300—375 cc of a 20 % alcohol solution. They observed, moreover, a marked dissociation between the tactile and the pain senses, the same dose of alcohol being without effect on the tactile sensitivity.

HARDY, WOLFF and GOODELL (1940) used another principle to determine the pain threshold. The light of a 1 000 W lamp was focused through an aperture onto the forehead of the subject for three seconds. The intensity of radiation was increased until the subject just felt pain at the end of the exposure. The intensity was ascertained in g cal/sec/cm<sup>2</sup> by means of a radiometer placed in the aperture. After ingestion of 30 to 60 cc of alcohol

the pain threshold increased by 30 to 50 % (WOLFF, HARDY and GOODELL 1941). Even these observers emphasized the difference between the tactile and the pain senses.

Changes in sensitivity thus seem to form part of the influence of alcohol on sensory functions. Earlier experiments on animals (NEWMAN and CARD 1937 a, GOLDBERG and STÖRTEBECKER 1941) suggested the sensitivity of the cornea to touch to show variations corresponding to the alcohol effect, the blinking reflex used as criterion of the effect. These variations were, however, difficult to ascertain quantitatively. No quantitative investigations in man on the changes of corneal sensitivity due to alcohol seem to have been carried out.

ZWAARDEMAKER and LANS (1899), experimenting on animals and man, used a puff of air blown against the cornea as sensory stimulus. For the experiments on man to be described the following device (fig. 7) was worked out to prove the suitability of applying corneal sensitivity as a quantitative measure of the alcohol effect.

## Experiments.

### Technique.

The subject (1) sat with his head in a chin-rest (2) and was instructed to look straight ahead and not to blink frequently. The air stimulus was directed to the eye of the subject through a glass tube (4), firmly attached to the stand of a magnifying tube<sup>1</sup> and placed exactly opposite the centre of the cornea by adjusting the stand. The distance between vertex corneae and the glass tube was usually kept constant at 62 mm. This was done by setting the cornea [(1) in fig. 7 B] at one end of a scale (10) in the magnifying tube, while the glass tube was set at the other end [(4) in fig. 7 B]. The length of the scale corresponded to a distance between the glass tube and the cornea of 62 mm by means of a negative lense of — 40 D, inserted between the objective of the magnifying tube and the glass tube.

The pressure was produced by means of an ordinary rubber balloon (6) from a blood pressure apparatus, closed by a valve (5). The height of the pressure was measured on a methylen jodide manometer [(8) LILJESTRAND and ZANDER 1928], allowing

<sup>1</sup> From Fa. Zeiss, Göttingen.



of readings with an error of  $\pm 0.5$  mm Hg. On releasing the pressure by pressing the valve, a puff of air was blown through the glass tube onto the eye.

The strength of the puff of air acting on the cornea will depend on the amount of the pressure, the volume of the pressure

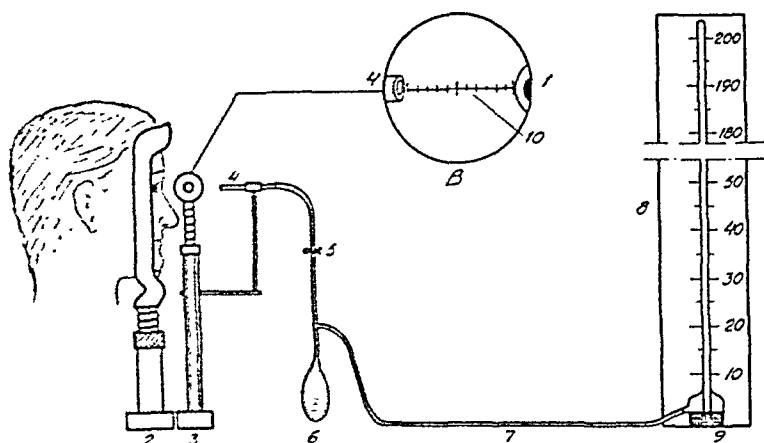


Fig. 7. Device for ascertainment of sensitivity of the cornea to air stimuli.  
(Explanation see text.)

vessel (9), the length and diameter of the rubber tubes (7) connecting the glass tube with the manometer, the inner diameter of the glass tube, the time needed for releasing the valve, and finally, the distance between the glass tube and the eye.

The total volume of the system: manometer to valve was  $50 \text{ cm}^3$ , the volume from the valve to the outer edge of the glass tube  $4 \text{ cm}^3$ , the diameter of the glass edge 3 mm. By keeping all factors constant and only varying the height of the pressure, comparable values were obtained on one and the same individual during an experiment.

The *critical value* was found in the following manner:

The determination was started by subliminal stimuli so as to control that the subject did not react to the possible noise of the air puff etc. The height of the pressure was then increased until the puff of air elicited a blinking reflex. After that the pressure was varied with alternating subliminal and supraliminal stimuli. The pressure in mm Hg that gave blinking in four trials out of five was taken as the critical value. In all some fifteen to twenty readings were made for each determination.

The size of the blinking reflex varied somewhat from individual to individual. Generally the first apparent beginning of the blinking of the upper lid was taken as the criterion, and this was kept constant in one and the same subject throughout the whole experiment. A suitable deflection was easily ascertained by a few preliminary trials.

The criteria described above were kept as standard conditions. In an early series of experiments (p. 14) a distance of 20 mm was fixed between the glass tube and the cornea, and a glass tube with a diameter of 1 mm was employed. In these cases the lense had to be substituted by one of — 7 D in order to keep the setting on the scale constant.

*Evaluation:* The height of the pressure expressed in mm Hg was used as a measure of the effect. The logarithmic mean of the first two determinations served as a basal value (p. 16). The values following after alcohol ingestion were likewise transformed to logarithms, and the logarithmic difference between the value of a trial and the basal value (p. 16) was plotted against time and against blood alcohol.

No experiments were made to calibrate the effect on the cornea in absolute units or to study more extensively the possible relationship between the height of the pressure and the distance and diameter of the glass tube, nor the possible difference between the right and the left eye. The right eye was used throughout the experiment.

## Results.

The test was very easy to perform and required no cooperation from the part of the test subject. A typical series of readings is shown:

$$15-18 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 20 \pm 22 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 25 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 28 + 30 + +.$$

Critical value = 25 mm Hg.

Some people, however, reacted already at six to eight mm of pressure, not only with a blinking reflex but with contraction of all muscles, so that they almost jumped from their seats. The test could not be used in these cases, as no consistent values could be obtained, and they were not included in the mean (3 subjects out of 37).

The proof that the method used is a test of corneal and not of lid

sensitivity was shown by anesthetizing the cornea. After corneal anesthesia no lid reflex could be elicited, only a slow, "worm-like" movement of the lower lid was sometimes seen. As criterion of the corneal sensitivity the blinking of the *upper* lid was therefore taken.

The range of pressure necessary to elicit a blinking, *i. e.* from the first indication of a blinking to an apparent one, varied from individual to individual. In the majority of cases this range was only three to five mm Hg, sometimes it could cover as much as ten to fifteen mm Hg. By making a few preliminary trials the special reaction of a subject to the puff of air was tested and a suitable criterion found. Usually the first blinking movement of the upper lid was used as a criterion throughout the whole experiment.

The range for 0 to 100 % response of one and the same criterion likewise varied from individual to individual. Some tentative tests showed the response curve to be approximately a probability function. For practical reasons the 80 % response and not the 50 % was used (p. 40). If the method is to be used for ascertaining only one value in an individual and not to perform a series of tests, it is advisable to ascertain the whole response curve by making some thirty to fifty readings at different heights of pressure. The 50 % response is then calculated *e. g.* according to BLISS (1938). The range was 10—15 mm Hg.

In one or two of the normal cases an increased flow of tears was observed during a trial when more than twenty readings were made, which seemed to increase the value of the threshold by about 5 mm Hg. By making fewer readings, about ten in these cases, this source of error was eliminated. No irritating dryness of the cornea was observed.

In a few cases it was seen that the threshold changed after some readings. It seemed as if the subject "inhibited" his reaction, the threshold being increased by five to ten mm Hg. Even this reaction was easily found out by the preliminary trial, and during the experiment the same number of readings was always used.

### Basal Values.

Basal values were determined in 18 subjects with the standard technique and were 29.9 mm Hg. The mean log basal value was  $1.476 \pm 0.0458$  (table 5).

The log variation of a single determination ( $\sigma_s$ ) was ascertained from 30 double determinations and was 0.0333, corresponding to 2.29 mm Hg or 7.66 % of the mean.

The log variation during the day ( $\sigma_d$ ) was determined from 12 control experiments and was 0.04276, corresponding to 2.94 mm Hg or 9.84 %. No influence of practice nor of fatigue was seen (fig. 8 ■—■).

The log variability from day to day was determined in 15 experiments on 7 subjects, *i. e.* in 8 double experiments, and was 0.0343, corresponding to 2.37 mm Hg, or 7.9 %.

Finally the log variation between individuals (18 subjects) was 0.1943, corresponding to 13.38 mm Hg or 44.74 %.

### Alcohol Experiments.

Alcohol ingestion brought about typical changes in the sensitivity of the cornea to air stimuli. Firstly the pressure of the puff of air necessary to elicit the blinking reflex, had to be increased. This indicates an increase in the pain threshold, as the cornea only has pain nerve endings. A typical series of readings is given:

$$35-40 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 45 \pm 50 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 52 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 55 \begin{smallmatrix} (+) \\ (-) \end{smallmatrix} 60 + 65 +.$$

Critical value 55 mm Hg.

Secondly the range between the 0 % and the 100 % response doses increased with the height of the critical value, and was found to be 15—30 mm Hg against 10—15 mm normally. No quantitative studies on the relation between the magnitude of that range and the height of the pressure were made. The experiments, however, suggest the percentage range to be rather constant.

An average curve, based on 14 experiments on 14 subjects, is given in fig. 8. The coincidence between the height of the critical value, in absolute values ( $\times - \times$ ) as well as in logarithms ( $\bullet - \bullet$ ), is obvious.

If the logarithmic differences, *i. e.* the degree of intoxication (p. 16) were correlated with the blood alcohol concentration, a rectilinear relationship was found (fig. 22  $\bullet - \bullet$ ).

The appearance and disappearance thresholds for the symptoms, computed as said before (p. 22) were 0.31 and 0.36 ‰ (table 6, col. 1—3); the difference is not significant. They were thus somewhat lower than the corresponding ones for brightness for fusion.

The slope of the regression line during the disappearance of the

symptoms was 0.3948, thus of the same magnitude as the slope for the brightness. But as the threshold value was lower for the corneal test than for the flicker test, the former shows a somewhat

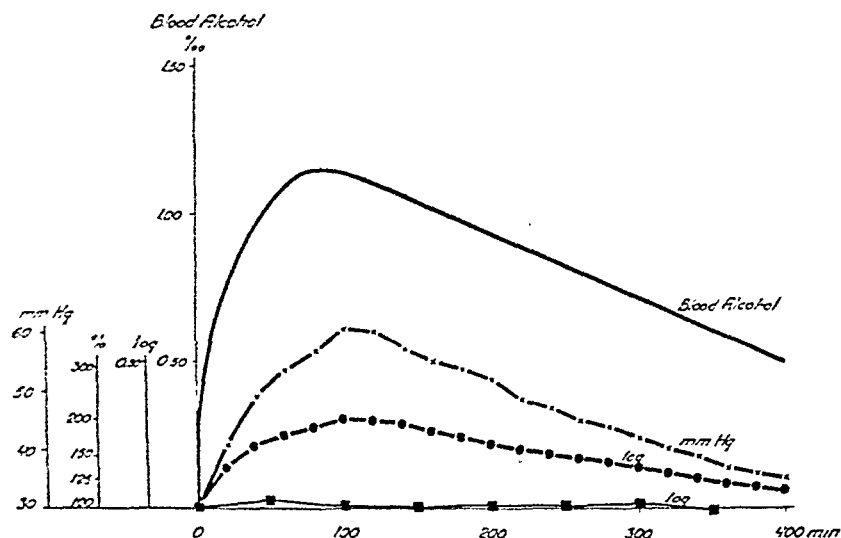


Fig. 8. Corneal sensitivity.

Changes during alcohol intoxication. Mean of 16 experiments.

- Blood alcohol curve.  
 ■ — ■ — ■ Deviations in control experiments (log diff.).  
 ● — ● — ● „ „ alcohol „ „ „  
 x — x — x „ „ „ „ (absolute values in mm Hg).

higher degree of intoxication at a given blood alcohol intoxication. At 1 ‰, for instance, the corneal test gives a degree of intoxication of 178 % against 151 % with the flicker test.

The experiments thus proved the threshold for pain sensitivity to rise with increasing blood alcohol concentration, as indicated by the increasing height of pressure necessary to elicit a blinking reflex. On account of the low threshold for the appearing and disappearing symptoms and the relative small variability, as compared with the deviations after alcohol ingestion, this test, as well as the flicker test, seems to be suited for following alcohol intoxication quantitatively.

## B. The Effect of Alcohol on Motor Functions.

### 1. Standing Steadiness (Quantitative Romberg Sign).

Disorders of neuro-muscular functions are observed among the early signs of alcohol intoxication, and alterations in station and gait are the most obvious. Their relation to disorders of cerebellar functions was early recognised. FLOURENS even in 1824 emphasized the resemblance between the staggering gait of the intoxicated person and that of a patient with a cerebellar disease.

Disturbance of station (the Romberg sign) and of gait have also been used to diagnostisize alcohol intoxication, *e. g.* for medico-legal use after traffic accidents etc. Attempts to record and to evaluate these clinical tests quantitatively were, however, only made to a small extent.

IMMERMANN in 1865 gives one illustration of the swaying after taking alcohol. The record was made by a method referred to VIERORDT (1864). A writing point was attached to the head of the subject, and made a tracing of the sway on a smoked paper suspended over the point.

The same principle was applied by CARLSON *et al.* (1934), who used a similar method worked out by LEE and KLEITMAN (1923). They found a slight increase in the area of sway after small quantities of alcohol taken in the form of beer.

MILES (1922 b) constructed a mechanical means, the "ataxiometer", which automatically summed up all movements in terms of their antero-posterior and lateral components, thus giving the amount of sway. Graphic records were simultaneously made. MILES (1924) found this test to be a sensitive indicator of the effect of alcohol. Both the amount of sway and the scatter or area of sway increased from about 30 to 60 % after small quantities of alcohol. The recording of the amount of sway yielded more uniform measurements from period to period than the evaluation of the graphic record of the area of sway.

Thus the experience quoted along with experiments in animals (NEWMAN and CARD 1937 a, b, NEWMAN and LEHMAN 1938, GOLDBERG and STÖRTEBECKER 1941, 1943) and in man (HAMMARSTEN and LILJESTRAND 1922, MILES 1924) suggest cerebellar functions to be influenced even by small quantities of alcohol.

On the other hand it has been reported that in some cases a clinical examination may fail to reveal any disturbance of cerebellar functions even at high blood alcohol concentrations (ANDRESEN 1938, LILJESTRAND 1940). This was partly ascribed to the patient's "ability to pull himself together" for the moment of examination and afterwards to relapse into the intoxicated stage again (ANDRESEN 1938).

This discrepancy between clinical examination and blood alcohol concentration favours the desirability of recording objectively *e. g.* the Romberg sign. Referring a quantitative measurement to blood alcohol during the course of intoxication in an individual will make it possible to get at the relationship between symptoms and the blood alcohol level. Moreover, it would make a decision possible as to whether the discrepancy is due to inaccuracies of clinical examination or whether there really exists a difference in tolerance between individuals in this respect. Statistical analyses show that different observers have varying criteria of diagnosis, which allow of a great range of subjective judgement (ANDRESEN 1938, HUBER 1938, LILJESTRAND 1940, DAHLBERG 1941.)

Preliminary experiments have shown that the Romberg sign gives such great deviations after alcohol ingestion in people unaccustomed to alcohol that the classic methods of VIERORDT or of MILES would have failed to record them. — HOLMES (1917, 1939) and TALPIS (1928) recorded cerebellar disorders by attaching small lamps to *e. g.* the finger tips, and then photographed the movements in a dark room. This principle was adopted for recording the Romberg sign.

### Experiments.

The method of recording the Romberg sign consisted in photographing the deflections of a source of light attached to the subject while standing. Certain conditions had to be fulfilled to make this test suitable for practical purposes. The method must make it possible to take a series of records of all sizes, from the minute deflections under normal conditions to the gross movements of the heavily inebriated. Further, it must permit of quantitative evaluation.

The problem to be solved contained as its chief points: a. The device must be used in daylight, and nevertheless the only light to be seen on the film must be from the electric bulb attached to the subject. b. The light must be so strong as to give a tracing

during rapid deflections, *e. g.* when the subject is falling, and at the same time not so strong as to give considerable irradiation on the film while standing.

By using a miniature camera and eliminating the daylight firstly by means of an ortho-chromatic film, secondly by a small aperture and a combination of filters, thirdly by a 40 W electric bulb as a source of light, the conditions mentioned above were fulfilled and the following device was constructed.

### Technique.

The camera was a Leica III c, taking forty pictures,  $24 \times 36$  mm, and equipped with a "Telerewinder" which permitted rewinding and releasing at a distance. The camera was attached to a firm stand 3 meters from the ground.

The lense was LEITZ ELMAR  $f = 3.5$  cm, the aperture used  $f/15$  to  $f/18$ . It allowed of taking objects of  $66 \times 99$  cm at a distance of 1 m.

The filters used were a green one of glass and two Dufoycolor filters (gelatine).

The film employed was either an ortho-chromatic 35 mm Agfa "Documenten" film, ca. 13 Din, or Eastman Kodak Positive film ca. 10 Din. The exposure time was fifteen seconds.

A 40 W electric bulb, serving as a source of light, was fixed on top of an extendable bar and attached to a firm stand, which was placed on the shoulders of the subject. The electric bulb was constantly kept two meters from the ground by adjusting the extendable bar according to the length of the test subject, and was thus one meter from the camera lens. The light conditions of the room were of no importance.

The records were made with the subject in two positions, a. the ordinary Romberg position with feet together, eyes closed and hands at side. b. the modified Romberg position with one foot in front of the other, eyes closed and hands at side.

At the beginning of the experiment the subject made a few preliminary tests in order to get used to the device, which were discarded. He was asked to fixate an eye-mark at a distance, then to close his eyes and to try to keep up his position, first with feet together (ord. Rb), and then with one foot in front of the other (mod. Rb). Each position was held for fifteen seconds, with a few minutes' rest between. Generally one record of ord. Rb. and



one of mod. Rb. was made at each determination; in three experiments double determinations were carried out.

*Evaluation.* The size of the real area of sway was computed from the photographed area in the following manner:

The lens  $f = 3.5$  cm and the object at a distance of 1 m gave a linear diminution of 27.57 times. — The real area was thus  $(27.57)^2 = 760.103$  times larger than the photographed.

After development of the film the negative was enlarged in an ordinary enlarging apparatus, and the photographed area traced on a paper. The size of the tracing was determined by joining the other contours of the tracing and by measuring this area planimetrically by means of a planimeter.<sup>1</sup> The planimeter was adjusted so as to give the area in mm<sup>2</sup> as the sum of four turns.

The linear enlargement from the photographed to the traced area was 6.67 times. The real area was thus  $\left(\frac{27.57}{6.67}\right)^2 = 17.103$  times larger than the traced one.

For computation of the *logarithmic difference* no adjustment to real area was necessary; the traced area was transformed to logarithms, and the log difference obtained as usual (p. 16). The traced area could as well be transformed to absolute values, corresponding to the real area, by using the logarithmic values; then the adjusting factor of 17.103 was substituted by adding  $\log 17.103 = 1.233$ .

*Correction for irradiation.* As the source of light had to be rather strong in order to give good records during large deflections (p. 46), a certain amount of irradiation could not be avoided, which means that the photographed area is greater than that which corresponds to the real area. This occurred only with the resting lamp, i. e. in ordinary ROMBERG position (fig. 9 A, I, II and VII. The original film was more distinct than the reproduction).

The degree of irradiation depends on the relation between the intensity of the light and the size of the aperture of the camera. With a constant aperture and filter the irradiation increases with increasing intensity. Its size was ascertained in the following manner:

An absolute value of the area of sway was obtained by the principle of VIERORDT (1864): the electric bulb was substituted

<sup>1</sup> Fa. Conradi, Zürich, Switzerland.

by a point which during a ROMBERG position made a tracing of the swaying on a smoked paper. The area of this "smoke" tracing corresponded to the area of sway, and was used as a standard.

The same subject was then photographed, and the area of the photographed tracing on the paper (see above) referred to the "smoke" area. An example will illustrate the computation:

Size of "smoke" area  $303 \text{ mm}^2 = \text{antilog } 2.4814$ .

Size of tracing of photograph  $91.0 \text{ mm} = 1.9590$ .

log adjusting factor for transformation of the tracing to real area  $2.4814 - 1.9590 = 0.5224$ .

In order to adjust for possible variations in the intensity of the electric bulb, the procedure ought to be repeated from time to time by photographing the standard subject and comparing the photographed area with the standard. The irradiation was, however, not detected until a large part of the experiments had been carried out, and the series were adjusted for irradiation in the following manner.

The mean of a number of basal values from different individuals (about 100 trials on 25 subjects) were referred to the standard "smoke" area. The log adjusting factors so computed were  $+0.105$ ,  $+0.175$  and  $+0.491$  for the Summer, December and February series respectively, and were used to adjust irradiation of those photographed areas which showed irradiation to be seen on the film. The photographs showing visible deflections were not adjusted (cf. fig. 9 A, 1, 2, 7 and table 2).

The error of measure ( $\sigma_m$ ) was ascertained from the standard deviation of the differences between two measurements of one and the same area ( $\sigma_{d1}$ ) according to the formula (DAHLBERG 1940)

$$\sigma_m = \frac{\sigma_{d1}}{\sqrt{2}} \quad . . . . . (8)$$

$\sigma_m$  was  $3.91 \text{ mm}$ , corresponding to  $1.62 \%$  for areas less than  $250 \text{ mm}^2$ , (189 determinations) and  $12.05 \text{ mm}$ , corresponding to  $1.2 \%$ , for areas of  $250\text{--}25\,000 \text{ mm}^2$  (76 determinations).

The tracing error ( $\sigma_t$ ) was ascertained as above from two tracings and measurements of one and the same film at two different times. Its magnitude, determined from 36 double tracings, was  $28.7 \text{ mm}^2$ , which corresponds to  $8.76 \%$ .

## Results.

### Ordinary Romberg Position.

#### Basal values.

The ordinary ROMBERG position was rather easy to keep, most subjects not swaying obviously.

Basal values and their standard deviation are given in table 5.

The standard deviation of a single determination ( $\sigma_d$ ) was ascertained from 39 double determinations and was  $[0.0261] = 18.1 \text{ mm}^2$ .

The variability of the test during a whole day ( $\sigma_d$ ) was ascertained in 12 control experiments (p. 15) and was  $[0.1127] = 78.2 \text{ mm}^2$ . The increase in variability is most probably due to the influence of fatigue. The mean was not altered during the day (fig. 10 ■—■), which can be interpreted as the possible counteracting effect of practice on fatigue.

The variation from day to day in one and the same individual ( $\sigma_{dd}$ ) was found to be  $[0.0987] = 68.5 \text{ mm}^2$ , ascertained from 13 double experiments.

#### Alcohol experiments.

The changes in station after alcohol ingestion were often seen quite easily with the naked eye. The swaying had as a swinging centre the talo-crural joint, and in the case of higher alcohol dosage it increased to such an extent that the subject sometimes lost his balance and fell.

The subject himself was often absolutely sure that he was performing a better test than under normal conditions. Even if he observed his own swaying or fall, he felt sure he would make a better result next time.

The ROMBERG test was largely influenced by personal factors, consistent values only being found if the subject did his utmost at every trial. If he was careless and did not concentrate on his task, widely diverging values would be found. Only a few trials, about ten to fifteen out of some twelve hundred determinations had to be discarded on account of this.

The area of sway photographed and measured as said above, (p. 48), was a good measure of intoxication. A typical experiment is illustrated in fig. 9, and the corresponding values are given in table 2.

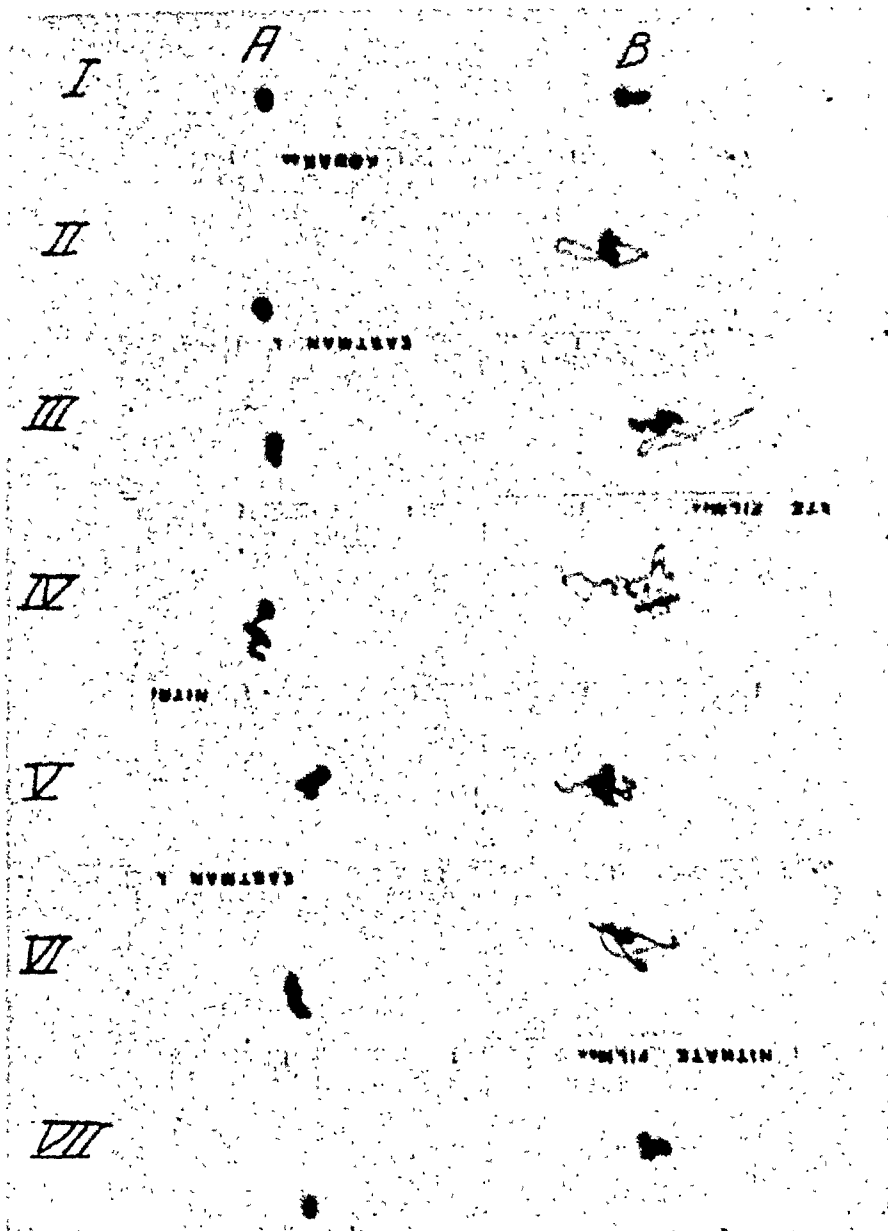


Fig. 9. Standing steadiness (quant. Romberg).

Variations in area of sway in alcohol intoxication (orig. size of film),  
9.2.43. Exp. 19 M. 1.0 g alc. per kg.

A. Ordinary Romberg position.

B. Modified Romberg position.

I. Before alcohol ingestion.

II—VII. After alcohol.

(Time, size and blood alcohol see text and table 2.)



The table also shows which areas had to be adjusted for irradiation.

The course of alcohol intoxication, plotted against time, is illustrated in fig. 10 by an average curve based on experiments

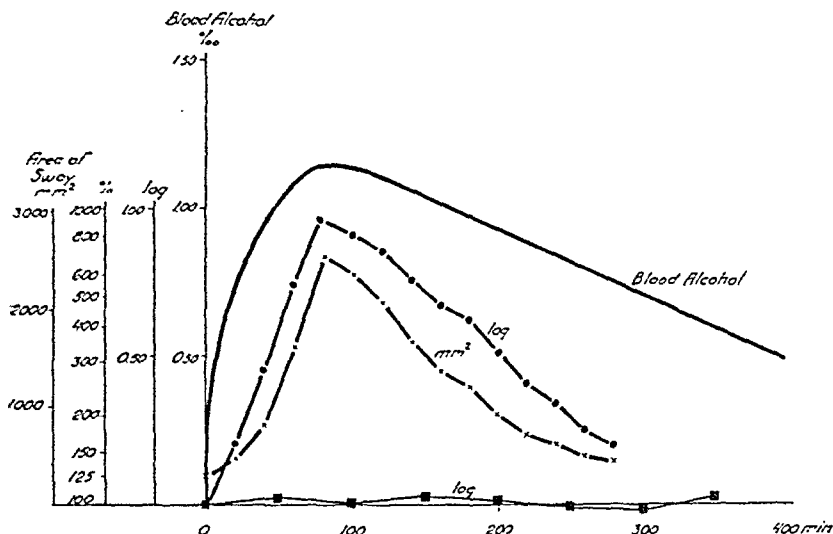


Fig. 10. Standing steadiness (ord. Romberg position).

Changes during alcohol intoxication. Mean of 17 experiments.

- Blood alcohol curve.
- — ■ — ■ Deviations in control experiments (log diff.)
- — ● — ● „ „ alcohol „ „
- x — x — x „ „ „ „ (absolute values in mm²).

on seventeen subjects. The coincidence between the area of sway, whether plotted in absolute values (mm²) or in logarithms, and the blood alcohol curve is obvious. The size of the area increased with increasing blood alcohol concentration, the maximum occurred approximately at the same time as the blood alcohol maximum, and the area then decreased with the fall in blood alcohol to reach the basal value before the blood alcohol had returned to normal. The deflections were great: the maximum degree of intoxication corresponded to 900 ‰.

Referring the degree of disturbance to blood alcohol (fig. 23 ● — ●) during the decreasing part (p. 22) gave a straight relationship, when log differences were plotted against blood alcohol concentration. The appearance and disappearance thresholds for the symptoms were 0.65 and 0.66 ‰ (table 6, col. 1—2).

The steep slope of the curve indicated that the function tested

was very sensitive to the influence of alcohol, the degree of intoxication augmenting at a high rate with a slight increase in blood alcohol. The degree of intoxication at a blood alcohol concentration of 1 ‰ was 490 ‰.

The individual thresholds were also ascertained graphically from individual curves (p. 22); the mean and stand. deviation are given in table 11, columns 4—6. The fact that the experimental error is smaller than the standard deviation (table 11, col. 9) indicates a real difference in threshold to exist between single individuals.

### Modified Romberg Position.

#### Basal values.

The changes in the standing steadiness with the modified ROMBERG position were more obvious than with ordinary ROMBERG position. A greater degree of swaying and a more frequent falling were observed, 14 subjects out of 40 showed obvious swaying already normally. The area of sway is illustrated in figs. 9 and 11 and in table 2, and the basal values are given in table 5.

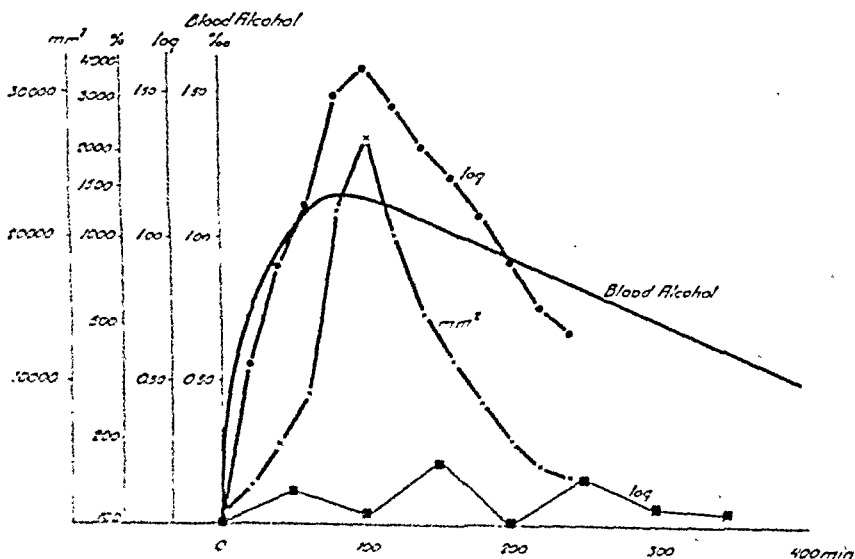


Fig. 11. Standing steadiness (mod. Romberg position).

Changes during alcohol intoxication. Mean of 17 experiments.

— — — — —	Blood alcohol curve.
■ — ■ — ■	Deviations in control experiments (log. diff.)
● — ● — ●	„ „ alcohol „ „
x — x — x	„ „ „ „ (absolute values in mm²).

The variability of this modification of the ROMBERG test (table 5) was greater than with the ordinary ROMBERG position. The great variability during the day is illustrated in fig. 11 (■—■).

### Alcohol experiments.

After alcohol ingestion the same principal features as with the ordinary ROMBERG position were seen. The deflections were, however, much greater. The course of alcohol intoxication as reflected by this test is given in figs. 9 and 11, and tables 2 and 6.

Referring the log differences to blood alcohol gives an approximately straight line (fig. 24). The appearance and disappearance thresholds were 0.41 and 0.65 ‰, suggesting this symptom to be more sensitive to the *rising* blood alcohol.

Its slope is steeper than that of the ordinary ROMBERG position which indicates this modification to be still more sensitive to the influence of alcohol than the ordinary ROMBERG position. The degree of intoxication at a blood alcohol concentration of 1 ‰ was 1622 %.

## 2. The Finger-Finger Test.

Next to disorders in standing and gait those of neuro-muscular in-coordination in the upper extremities are frequent after alcohol ingestion. A general method to record instability of neuro-muscular coordination has been that of letting the subject make dots with a pencil on a paper placed in front of him at arm's length. This method, in Sweden referred to BLIX (1883), was reported to show impairment in function of 30—50 % after so small a dose as 5 cc of alcohol in an abstainer (GYLLENSVÄRD 1918). Similar results were stated by VERNON (1919). YATES (1929) using a similar method of recording past pointing for diagnosis, found under normal conditions the dots to be "nearly superimposed"; in cerebellar or vestibular disorders the dots showed "a general instability or formed a line".

MCDUGALL and SMITH (1920) employed a "dotting machine" to reveal alterations in muscular co-ordination. The apparatus consisted of a band of paper tape drawn behind an opening. The test consisted in marking with a stylographic pen a series of small circles appearing in the opening at the rate of 5.8 a second in three to four minutes. After 10 to 25 cc of alcohol the per-



centage of errors rose from about 7 % to 8—14 % which means an increase due to alcohol of 20—100 %.

A labyrinth test, where the subject had to move a metal stylus within a boundary hoop along a line on a rotating plate between irregularly distributed spikes, was used by BAHNSEN and VEDEL-PETERSEN (1934). They found alcohol at a dose of 0.6 g per kg to impair the skill by increasing the number of errors by 35—80 %. Simultaneously blood alcohol analyses were run (SCHMIDT 1934), and the degree of disturbance correlated with the alcohol analyses. A correspondence between the impairment of function and blood alcohol was suggested.

CARLSON *et al.* (1934) tested hand steadiness by letting the subjects hold a metal stylus in a hole in a metal plate and counted the number of contacts during two one-minute periods. They found an increase of 10 % after four bottles of beer, corresponding to 54.6 cc of alcohol, at a blood alcohol concentration of 0.60 ‰.

The frequent use in the clinic of the simple finger-finger test which proves valuable to detect gross disorders of cerebellar origin inclusive those in alcohol intoxication, gave the idea of applying it for a quantitative study of alcohol intoxication.

## Experiments.

### Technique.

A disc 8.8 cm in diameter with a paper attached to it was kept firmly on the left forefinger by means of tubes of varying diameters, inserted in a holder and fitting the finger. A thimble with a point on it was placed on the right forefinger. (The disc and the thimble, of four different sizes, must fit so well that the forefinger tips were pressed against the bottom.)

The subject was sat at ease in the room, and was asked to close his eyes and instructed to try "to get his finger tops meet", *i. e.* not to try to mark the centre of the disc. The distance between the fingers when beginning was 40—50 cm. The dotting was repeated 50 times to the beats of a metronome, going 75 times per min. The subject had first a "practice" trial, which was discarded, in order to get used to the method, then he made two trials of 50 dots each.

*Evaluation:* As a measure of the degree of co-ordination, the area covered by the dots was used. The size of this area was deter-

mined by joining the outer dots by tracing, a biased dot when single being omitted, and by measuring this area planimetrically.

The planimeter was adjusted so to give the area in  $\text{mm}^2$  as the sum of four turns. The measurement was mere in two steps, each of two turns, and the total area was thus the sum of the two steps.

The error of measure ( $\sigma_m$ ) was determined from the differences between the two measurements ( $d_1$ ) of one and the same area, according to the formula given by DAHLBERG (1940):

$$\sigma_m = \pm \sqrt{\frac{S(d_1^2)}{2(n-1)}} \quad \dots \dots \dots (9)$$

$\sigma_m$  was  $\pm 4.568 \text{ mm}^2$  (310 determinations), corresponding to a coefficient of variation of  $\pm 2.11 \%$ .

The error of tracing ( $\sigma_{tr}$ ) was determined from double tracings by placing two papers on the disc and tracing and measuring them on two different occasions. The differences found ( $d_2$ ) gave the total variation ( $\sigma_t$ ), the error of tracing ( $\sigma_{tr}$ ) was computed from current formulae (DAHLBERG 1940):

$$\sigma_{tr} = \pm \sqrt{\sigma_t^2 - \sigma_m^2} \quad \dots \dots \dots (10)$$

$\sigma_t$  was  $\pm 16.33 \text{ mm}^2$  (30 determinations), corresponding to a coefficient of variation of  $\pm 2.58 \%$ .

$\sigma_{tr}$  thus became  $\pm 1.49 \%$ .

## Results.

### Basal values.

Normally the scatter of the dots was rather small, 80—90 % of the dots confluating. If the subject did not concentrate on his task, on account of fatigue or for some other reason, the dots deviated somewhat, about 50—70 % confluating and the others being seen as discrete holes in the paper, fig. 12 (1).

The average basal value (37 subjects) was  $[2.300] = 199.5 \text{ mm}^2$ .

The different variations:  $\sigma_s$ ,  $\sigma_d$ ,  $\sigma_{dd}$ ,  $\sigma_i$  are found in table 5, cols. 4—15.

The influence of practice and fatigue increased the variation but did not alter the area of dotting, as illustrated by the control experiments in fig. 14 (■—■).

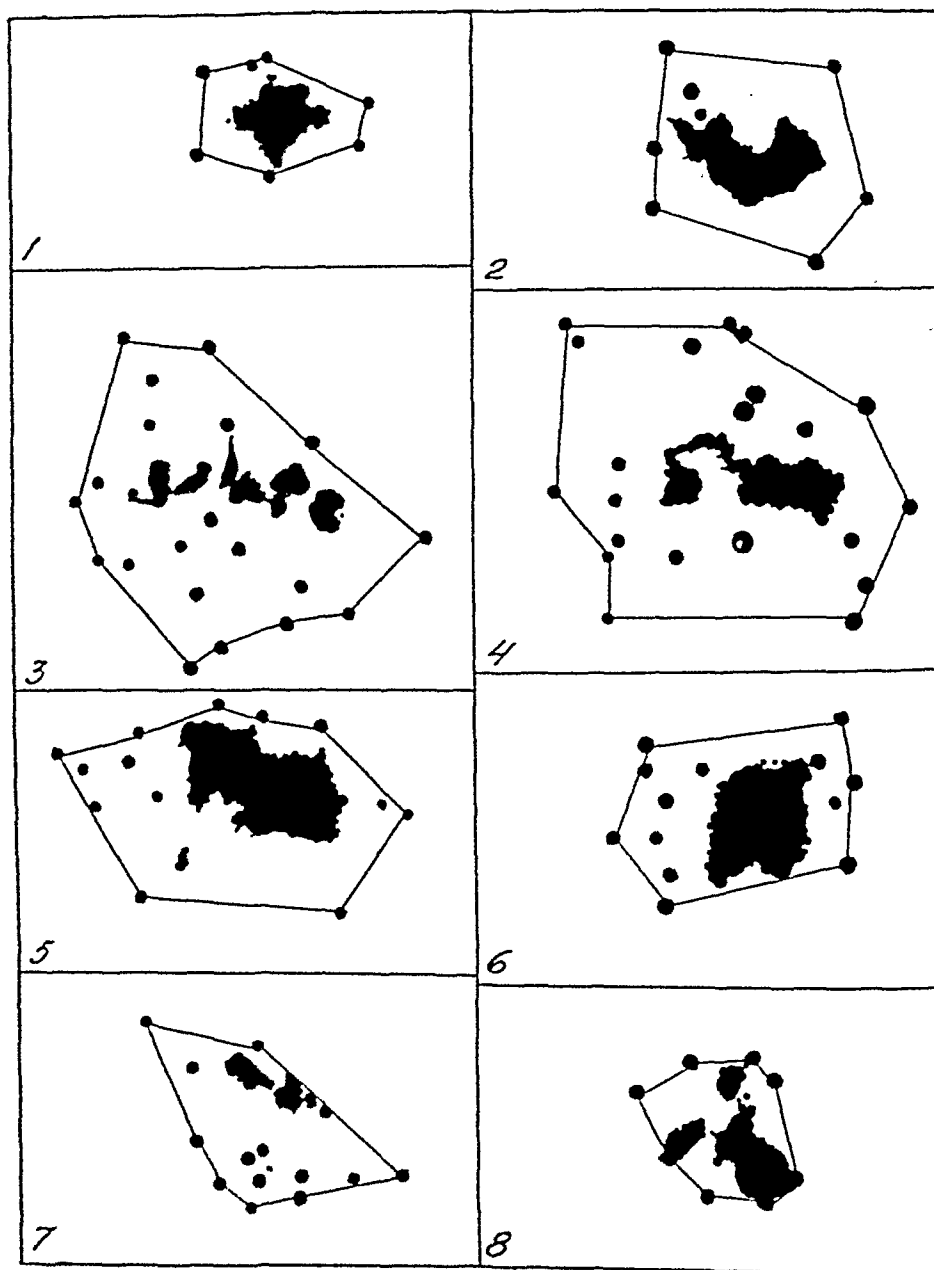


Fig. 12. The finger-finger test.

Variations in area of dotting in alcohol intoxication (Orig. size).

15. 12. 42. Exp. 15 M. 1.0 g alc. per kg.

1. Before alcohol ingestion.

2-8. After alcohol.

(Time, size and blood alcohol see table 3.)

**Alcohol Experiments.**

Usually the subjects had a feeling that they were performing the test better after alcohol than normally and were very astonished on being shown the result. This kept their interest in the task awakened as they tried to compete with themselves and yielded maximum values. On the other hand, when seeing a rather good result after alcohol the subject got sure of himself, his interest slackened and the second trial was less satisfactory. As a rule they were not shown their results. Sometimes the subjects showed difficulties in following the beats of the metronome, some of them ascribing this to their hearing the beats of the metronome less than before taking alcohol.

The tests showed typical departures from normal. The dots deviated more from the centre: a greater number became discrete, and the area of dotting increased. A typical case is illustrated in fig. 12 (2—7).

The figure clearly shows the increased scatter of the dots after alcohol, and the increase in area.

**Table 3.***Finger-Finger Test.*

14. 12. 1942. Subj. 15 M. 78 kg. 1.0 g. alc. abs. per kg at 9.40 a. m.

Number	Time after ingestion min.	Blood Alc. Conc. ‰	Area of Dotting		
			1st Trial mm <sup>2</sup>	2nd Trial mm <sup>2</sup>	Mean mm <sup>2</sup>
I . .	0	—	218	202	210
II . .	0	—	240 <sup>1</sup>	240	240
III . .	20	1.08	501 <sup>2</sup>	542	521
IV . .	57	1.22	978	904 <sup>3</sup>	941
V . .	82	1.32	972	1 137 <sup>4</sup>	1 050
VI . .	110	1.28	834 <sup>5</sup>	729	782
VII . .	180	1.10	585	479 <sup>6</sup>	532
VIII . .	234	0.97	440	365 <sup>7</sup>	403
XI . .	393	0.59	273 <sup>8</sup>	294	284

(1—8 correspond to the same numbers in fig. 12.)

In table 3 the corresponding data for time, blood alcohol concentration and area are given.

The table gives an understanding of the variation between the

two normal values (I and II), and between the first and the second trial. This latter difference seemed to vary with the size of the area.

If the difference between the two trials was plotted against their mean, and this was done with a great number of trials,

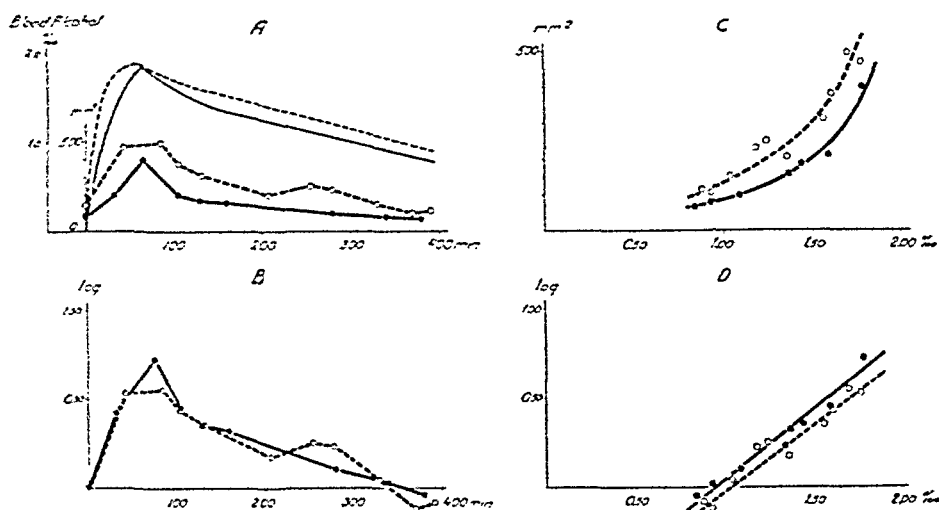


Fig. 13. The finger-finger test.

Variations in area of dotting in repeated experiments after alcohol ingestion.

- |    |                                      |                       |
|----|--------------------------------------|-----------------------|
| A. | Area of dotting in mm <sup>2</sup> , | plotted against time. |
| B. | " " " " log. differences             | " " "                 |
| C. | " " " " mm <sup>2</sup>              | " " blood alcohol.    |
| D. | " " " " log. differences             | " " "                 |
- 
- |       |                          |           |                       |
|-------|--------------------------|-----------|-----------------------|
| —     | Blood alcohol 27.3.42.   | Exp. 2 H. | 1.2 g alcohol per kg. |
| - - - | " " 18.6.42.             | Exp. 3 H. | 1.3 g alcohol per kg. |
| ● — ● | Area of dotting 27.3.42. |           |                       |
| ○ — ○ | " " 18.6.42.             |           |                       |

an increase of the difference with increasing mean was found. If the percentage difference between the two trials, or the logarithmic difference, was plotted against the mean, a horizontal course would imply the percentage or the logarithmic difference to be constant and independent of the magnitude of the mean. CROZIER *et al.* (1940) have suggested such a relation to exist for the flicker frequency of the eye, and have discussed its biological significance.

These calculations were tentatively carried out on 68 differences under normal conditions and on 219 differences after alcohol. The result agreed with expectation: the percentage difference was constant and about 15 % for both the normal and the alcohol series.

The reproducibility of the test and the application of absolute and logarithmic values is illustrated by a case, given in fig. 13.

In fig. 13 A the absolute values of the area of dotting, expressed in  $\text{mm}^2$ , were plotted against time. Both curves followed the course of the blood alcohol curve, but they differed from each

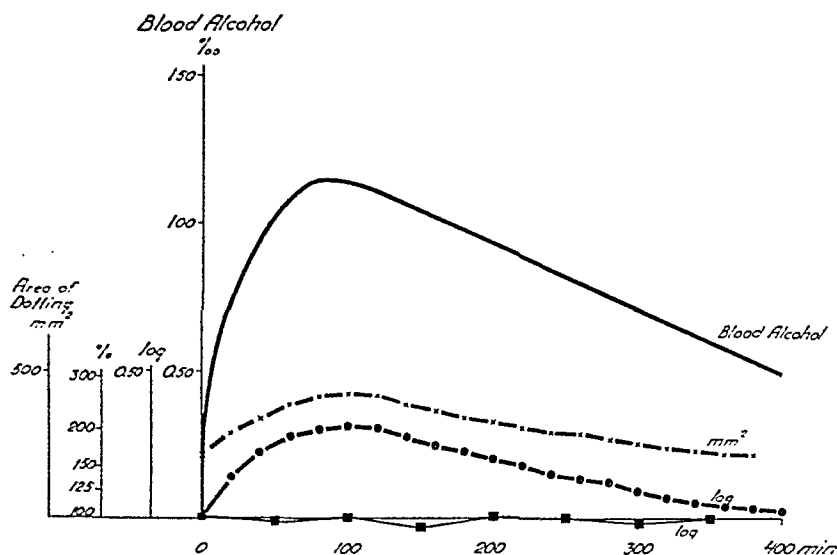


Fig. 14. Finger-Finger test.

Changes during alcohol intoxication. Mean of 17 experiments.

- Blood alcohol curve.  
 ■ — ■ — ■ Deviations in control experiments (log diff.)  
 ● — ● — ● „ „ alcohol „ „ „  
 x — x — x „ „ „ „ (absolute values in  $\text{mm}^2$ ).

other with regard to the height of their first values, which were 95 and  $140 \text{ mm}^2$  respectively.

In fig. 13 B the curves were drawn as logarithmic differences (p. 16), *i. e.* the differences between the logarithms of the values found and the basal value, and plotted against time. The curves now coincided within the range of error.

The relation between symptoms and blood alcohol was established in fig. 13 C, where the absolute values of the symptoms during the decreasing part of the curve were plotted against the blood alcohol concentration. The relationship seemed to follow an exponential curve, and the curves differed from each other. — In reality the difference was not so large as seen on the graph, as fig. 13 C was drawn in a scale twice that of fig. 13 A. The graph

further shows the difficulty to determine the decrease threshold from the exponential curve.

Finally the log differences for the symptoms were plotted against blood alcohol in fig. 13 D. The relationship became rectilinear, and the two regression lines practically coincided. This suggests that the relationship is rather constant from experiment to experiment in one and the same individual.

The same observations were also made with the other tests applied on several subjects. The number of experiments was, however, too slight to allow of quantitative statistics as to the variation from time to time.

In fig. 14 an average curve of the changes in the finger-finger test after alcohol, based on 19 experiments on 17 subjects, is given. The coincidence between symptoms and blood alcohol is obvious. In fig. 25 (● — ●) the relationship during the decreasing phase of the symptoms is illustrated, and values for the increase and decrease thresholds are given in table 6. There was a difference found between the appearance and the disappearance thresholds for the symptoms.

Observers in this field agree that the motor functions seem to be influenced to a high degree by alcohol (MELLANBY 1920, HAMMARSTEN and LILJESTRAND 1922, MILES 1924). The tests described, the photographic method of recording standing steadiness and the quantitative finger-finger test, yielded good results after alcohol, as indicated by the large departures from normal, recorded by them. The deflections, especially when using the test on standing steadiness were of a magnitude, which by far exceeded the results found by other observers: at a blood alcohol concentration of 1 ‰ the two variations of the standing steadiness test (ordinary and modified ROMBERG position) showed a degree of intoxication of 499 and 1,622 ‰ respectively, and the finger-finger test 174 ‰.

## C. The Effect of Alcohol on Psychological Functions.

The number of tests devised to study the influence of alcohol on psychological functions is considerable. The aim of this work, as far as it concerns psychological tests, will be primarily to investigate whether they permit a quantitative measurement of the

alcohol effect, secondly, to find the possible relationship between the degree of disturbance and blood alcohol, and finally, to compare the results obtained with psychological tests with those of sensory and motor functions.

The two classic tests in this respect are the KRAEPELIN test and the BOURDON test.

### 1. The Subtraction Test.

The KRAEPELIN test consists of continuously adding two digits, the number of additions and errors per time unit being evaluated. KRAEPELIN *et al.* (1892, KÜRZ and KRAEPELIN 1901) found a slight decrease in the number of additions and an increase in the number of errors after alcohol ingestion. FRANK (1925) and RIEGEL (1925) used this test to investigate the after-effects of alcohol and found a slight impairment still remaining the day after ingestion. HAAS (1925) employed the KRAEPELIN test to measure the depth of sleep, and performed additions for ten minutes after being awakened. After previous alcohol ingestion the performance deteriorated, this indicating a heavier sleep due to alcohol.

DANGER (1939) applied the addition test in experiments on the relation between blood alcohol and alcohol effect. He found a decrease in the number of additions by 10 to 20 % at a blood alcohol concentration of 0.8 to 1.2 ‰.

Psychiatrists often use a *subtraction* test to prove the ability of their patients to count backwards orally and they let them begin at 100 and go on subtracting 3. The test is rendered more difficult by subtracting 7, this modification seems to differentiate more (ODSTEDT, pers. comm.). Preliminary experiments showed that the subtraction test with 7, when evaluated by measuring the total time needed for fourteen subsequent subtractions, gave somewhat better results than an addition test. The following technique was adopted for the final experiments.

## Experiments.

### Technique.

The subject who sat in a closed room with no disturbing noise from outside, was asked to subtract 7 as quickly as possible, starting from 100 and going down to the least positive figure.



The time for the whole test was noted. The first five trials were made for practice and discarded. Each determination consisted of two trials, the subject always subtracting seven but each time beginning at a new number, which ranged from 99 to 104.

*Evaluation:* The total time in hundredths of minutes, necessary for the whole calculation, was used as a measure. The number of errors, normally varying between 0 and 4, was too small to permit of a quantitative evaluation and was recorded but not evaluated. — The total time found was transformed to logarithms and the value of the logarithmic difference between the value found and the basal value (see below) was plotted against time and blood alcohol.

## Results.

### Basal values.

In most subjects this test involved a good deal of mental strain as people generally are not used to this special kind of mathematical operation. Starting the calculation with the prime numbers 99 to 104 prevented the subjects from using the multiplication table backwards.

The functions which are tested will be concentration and attention, as well as memory and the ability of mathematical calculation. As other psychological tests this, too, was easily influenced by outer factors. A slight decrease in concentration momentarily increased the total time, which was often seen at the end of a day's experiments. On the other hand in "inhibited" subjects, disinhibition resulted in a shorter time. As compared with somatic functions, this test was much influenced by practice and fatigue, and more than the BOURDON test.

The effect of practice was most obvious on those subjects with long total times. It was noticed that part of the long total time in subjects of normal intelligence was not due to any mathematical inability but rather to inhibiting factors. These play their part primarily in the first trials, and are partly eliminated when the test has been practised a few times. Similar experience was reported by ARGELANDER (quoted from KURKA) and KURKA (1928).

The quantitative influence of practice and fatigue was ascertained as usual in control experiments on twelve individuals,

repeating the test nine times during six to eight hours. The average curve is illustrated in fig. 15 (■—■) and shows a slight but unmistakable improvement due to practice, the total time for the test decreasing from 0.389 minutes to 0.334 minutes on an average, corresponding to an improvement of 16.5 %. The influence of practice on the first trials was still more. This was the reason for discarding as many as five preliminary trials. Generally only the second normal value previous to the taking of alcohol was used as a basal value, even the first one being discarded to eliminate part of the effect of practice. On the other hand, fatigue very easily prolonged the total time of the performance, it was sometimes seen that in a few cases the values at the end a day were higher than those at the beginning. This was especially noticed after alcohol, and above all if the subject was sleepy.

The basal value (mean of 37 subjects) was 0.431 min.

The variation of a single determination ( $\sigma_s$ ) could not be calculated as usual, as only one determination was made before the alcohol was taken.

$\sigma_s$  was tentatively computed in another manner: As each determination was the mean of two trials, the difference between these two trials was plotted against the mean. The difference rose with increasing mean (like the finger-finger test p. 60). If the *percentage* difference was plotted against the mean, an approximate horizontal line was found, suggesting the percentage difference to be constant, and independent of the magnitude of the mean. The percentage difference ( $d_{\%}$ ) was graphically computed to be about 18 %, and the stand. dev. of a single trial  $\left(d_{\%} \times \frac{1}{\sqrt{2}}\right)$  thus about 12.7 %, and the standard error of the mean  $\left(12.7 \times \frac{1}{\sqrt{2}}\right) = 9.0$  %.

This figure agrees well with the variation during the day, which was found to be 12.11 %, and from day to day = 18.11 % (table 5, col. 8, 11).

The variation between individuals was 37.30 %.

### Alcohol Experiments.

The effect of heavy alcohol dosage was very typical, the total time and the number of errors increasing. A typical series is given below: 104 97 90 83 66 59 52 45 48 41 34 27 20 13 6.

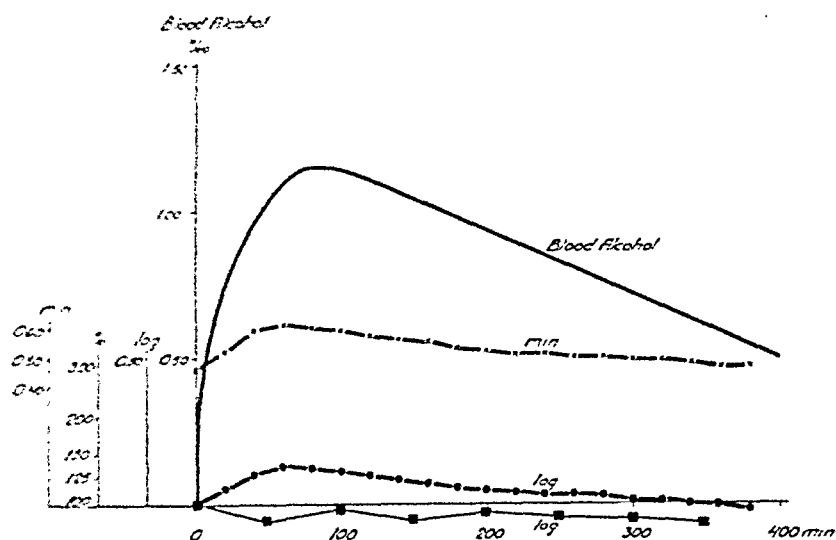


Fig. 15. Subtraction test.

Changes during alcohol intoxication. Mean of 17 experiments

- Blood alcohol curve.
- — ■ — ■ Deviations in control experiments (log diff.).
- — ● — ● „ „ alcohol „ „ „
- x — x — x „ „ „ „ (absolute values in min.).

The increase in the number of errors is not so great as to explain the prolongation of time. This must be due to other factors, a greater diversion of attention, lack of concentration, etc.

Smaller alcohol doses had principally the same effect but in a quantitatively minor degree. Sometimes it was so slight that practice seemed to conceal it altogether, since the total time was not altered. — Fatigue which also impairs the results, was sometimes seen to influence the performance of the test after alcohol to a greater degree than normally, giving an impairment in the last or last trial but one of the day.

The average of experiments on 17 subjects is illustrated in fig. 15, where absolute values ( $\times - \times$ ) and log differences ( $\bullet - \bullet$ ) are plotted against time.

It was obvious as well from the average curve as from the observations on a single individual that the maximum of effect occurred soon after the ingestion of alcohol had taken place, *i. e.* before the blood alcohol had reached its maximum height.

The reason for this might lie in two conditions. The one will be that the functions tested by this method are more sensitive to the rising of the blood alcohol than the level of blood alcohol as such. The other factor may lie in a compensative mechanism

which appears after the first effect of alcohol on the central nervous system has set in. The nature of this compensative mechanism is not settled, but may primarily lie in an increased effort of will-power, resulting in an increased amount of concentration and attention.

The symptoms were finally related to the blood alcohol concentration during the rising and the decreasing phase of the symptoms and the blood alcohol curve.

The decreasing phase is illustrated in fig. 26 (●—●).

The appearance and disappearance thresholds differed (table 6, cols 1—3), the appearance thresholds being considerably lower, which explained the higher degree of intoxication during the rising of blood alcohol. This was primarily due to the difference in thresholds (p. 84).

The subjects themselves claimed this test to be very difficult to perform. On the other hand the results showed, that the subjects were able to compensate much of the possible effect of alcohol by an increased mental effort.

The impairment of function with the increase of blood alcohol was slight, as indicated by the slope of the regression line, expressing the relationship between symptoms and blood alcohol (table 6, col. 5 and fig. 26).

Moreover, the influence of other factors than alcohol, as diversion of attention, fatigue, sleepiness etc. was of the same order of magnitude as that of alcohol, and could thus fully conceal any action of alcohol.

Finally, the effect of psychic compensation was considerable, but varied to a great extent. By forcing the subject to work at his maximum of effect, part of the psychic compensation could be eliminated; this was one reason, why conclusive values at all were obtained.

This method can only serve to detect gross disorders in psychic functions; it yields principally the same results as sensory and motor tests, but of another order of magnitude, both with regard to threshold values as to the magnitude of departures from normal due to alcohol.

## 2. The Bourdon Test.

The BOURDON test (BOURDON 1895) was applied as the second psychological one in this study. This test, first referred to OEHRN (1889), consists in its original form of a continuous text, in which

the subject has to cross or mark one or more given letters as quickly as possible during a certain period of time. The test is evaluated by counting *e. g.* the number or percentage of letters correctly marked, the total number of letters read, the number or percentage of errors, or the time needed for performing a certain paragraph etc. As a modification a text consisting of single letters, distributed at random, was introduced by SHARP (1899).

RIEDEL (1925) employed the BOURDON test during an period of 90 days of alternating control and alcohol experiments; alcohol caused an impairment of 17 to 19 %. KURKA (1928), studying the influence of different concentrations of alcohol on several functions, found with this test an increase in the number of errors of 25—35 % after taking alcohol. Finally, PETER (1939) in investigations concerning the influence of sedatives on the alcohol effect employed a BOURDON test to study the changes in psychological functions. The results of this test followed the blood alcohol curve to a certain extent, the maximal decrease in performance being about 20 % at a blood alcohol level of about 1 ‰.

A close study of a number of investigations in this field on widely different themes, *e. g.* psychological works concerning the effect of fatigue (GRAF 1925), of drugs as benzedrine (LEMMEL and HARTWIG 1939, ALWALL 1942), or as a test of selecting applicants (ANDERBERG 1935) etc., shows this test to be liable to a great variability. The degree of the subject's concentration as well as his interest in his task, the amount of will-power on the one hand and the diversion of attention on the other, and the influence of practice are some of the factors that cause the variability.

The character of this work made it necessary to use tests, which would keep the subject's interest awakened, as they had to be repeated several times, and as a number of different tests was employed. It was therefore primarily chosen to use some kind of a continuous text. This would keep the interest of the subject awakened and at the same time serve as a certain amount of diversion for attention and render the test somewhat more difficult. On the other hand the words must not be so easy as to permit the subject to recognise them without any difficulty. In fact the effort to *mark* letters was so strong that the subjects very seldom had any memory of the contents of the text.

Preliminary experiments also showed that at least three letters had to be marked in order to give consistent values. The final technique adopted is described below.

## Experiments.

### Technique.

Seven different Swedish texts were used. They were taken from a narrative of Swedish life in the sixteenth century, (by WIDÉN, Svenska Turistföreningens Årsskrift 1941) written in a somewhat archaic language, containing rather long and not very familiar words (fig. 16).

The subject, who sat in a quiet room, had to mark three given letters in four minutes every time they appeared in the text. The first letter was to be indicated by a stroke (/), the second by a cross (×) and the third by a circle (○).

The subject was instructed to mark all the three letters at one and the same time and not to take first one through the whole text, then the next one, etc., which would have made the test easier. He was also told to work as quickly as possible and to try to mark correctly as many letters as possible during the trial. It was not necessary for him to remember the letters as they were written and marked on the sheet of paper.

The letters for each text were selected in the following manner. The first two letters belonged to the seven most frequent letters in Swedish (a, e, l, n, r, s, t). The same letter was used for two different texts, once as first letter (/) and the next time as second letter (×). There were at least two texts before one and the same letter was used again. The third letter (○) was chosen among those letters which come next in frequency after the first seven mentioned.

The texts were so selected that the frequency of each of the first two letters was about the same and twice the frequency of the third letter. Letter combinations and the frequency of the letters used are given in table 4.

*Evaluation:* The relative degree of difficulty in the seven texts was tested in 560 trials on 194 persons in all, the texts being distributed at random in order to avoid any influence of practice or fatigue. The results are given in table 4.

The seven texts were practically of the same degree of difficulty. No adjustment for the slight differences was made, as the deviations from the mean were not significant. The standard deviation was  $\pm 30$  letters, the standard error of the mean  $\pm 2$  and the standard error of the difference  $\pm 3$ .

Table 4.  
*The Bourdon Test.*  
 Frequency of Letters and Relative Degree of Difficulty.

Text	Letters				Number of Trials	Number of Letters (N)		Relative Degree of Difficulty ( $1/N \times M \times 100$ )	
	Absolute Frequency			Total Number per Text		Correctly Marked (BC)	Totally Read (BT)	Be %	BT %
	1st (/)	2nd (x)	3rd (O)						
I . . . . .	a (73)	t (71)	b (31)	165	87	117.67	133.47	99.78	99.66
II . . . . .	o (70)	s (61)	d (44)	175	81	115.86	134.22	101.28	99.11
III . . . . .	l (63)	n (72)	f (31)	166	83	116.89	130.71	100.39	101.77
IV . . . . .	s (66)	r (77)	g (42)	185	83	122.46	136.78	95.83	97.25
V . . . . .	n (96)	a (95)	u (11)	202	80	118.99	138.49	98.62	96.05
VI . . . . .	t (64)	e (73)	v (21)	158	75	115.11	130.15	101.94	102.21
VII . . . . .	r (65)	i (59)	i (42)	166	71	113.80	128.17	103.11	103.78
Total 560					Mean (M)	117.35	M · 133.02	100 %	100 %

The texts were evaluated by counting

- a. The number of letters correctly marked = BC
- b. The total number of letters read = BT.

The mean of the first two trials served as a basal value (p. 16). The values found were transformed to logarithms and the logarithmic difference (p. 16) plotted against blood alcohol and time.

It was shown in a preliminary study that evaluating the percentage of correctly marked letters or of the percentage of errors = the percentage of incorrectly marked or omitted letters, gave less consistent values than using the number of correctly marked letters or the total number of letters read. Therefore only the two last mentioned measurements are included in this study.

### Results.

The performance of a trial was influenced by *e. g.* all the factors which normally divert attention, and a consistent performance of the test demanded a quiet room with no disturbance from outside, and definite and repeated instructions to the subject to concentrate on his task and do his best.

The instruction to work at top speed was necessary in order to get conclusive values. Otherwise the subject adopted an average rate of working which he kept rather constant by varying his concentration on the task. By the force to work maximally the effect of compensating an influence from outside by increasing the mental strain was lessened.

The time of performance of this test, four minutes, was enough for the subject to arrive at a normal degree of practice. This was reached after only one minute, and the amount of work performed during the remaining three minutes was rather constant. On the other hand four minutes under normal conditions were not enough to produce any visible signs of fatigue.

### Basal Values.

The basal values for the number of letters correctly marked (BC) and the total number of letters read (BT) are seen in table 5, cols. 2 and 3 (27 subjects). The figures cannot be considered as representative for an average population, as 24 of the 27 subjects were students; this test will be correlated with education to a certain degree.



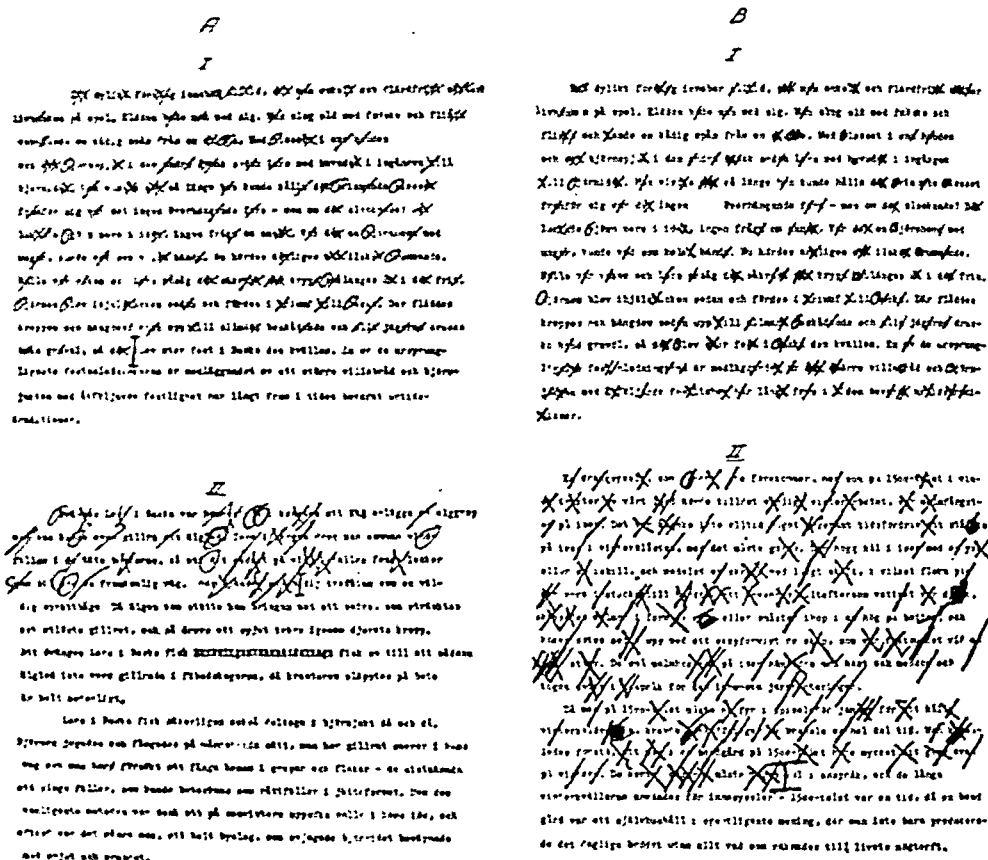


Fig. 16. The Bourdon test.

- A I. 18.6.42. Exp. 3 H. Before alcohol ingestion. Letters: a t b. Result: 116/127 in 4 min (BC/BT).
- A II. " " 45 min after alcohol ingestion. Letters: e s d. Result: 23/49 in 4 min (BC/BT). 1.88 ‰ alcohol.
- B I. 10.2.43. Exp. 10 A. Before alcohol ingestion. Letters: a t b. Result: 153/165 in 4 min (BC/BT).
- B II. " " 110 min after alcohol. Letters: n a u. Result: 145/161 in 4 min (BC/BT). 0.88 ‰ alcohol.

The values for the variability under different conditions are seen in table 5, cols. 4—15. The coincidence between the two methods of evaluation: BC and BT, is complete within the range of errors.

The variability during the day ( $\sigma_n$ ) was illustrated in figs. 17 and 18 (■—■). No significant change, due to practice, was

noted. On the other hand, the increase in  $\sigma_d$  as compared with  $\sigma_s$  must be attributed to the influence of fatigue.

### Alcohol Experiments.

The behaviour of the subject, when performing a trial under the influence of alcohol, was very typical. He talked to himself and could sit for seconds doing nothing. His handwriting became "atactic": the pencil markings grew larger and thicker,

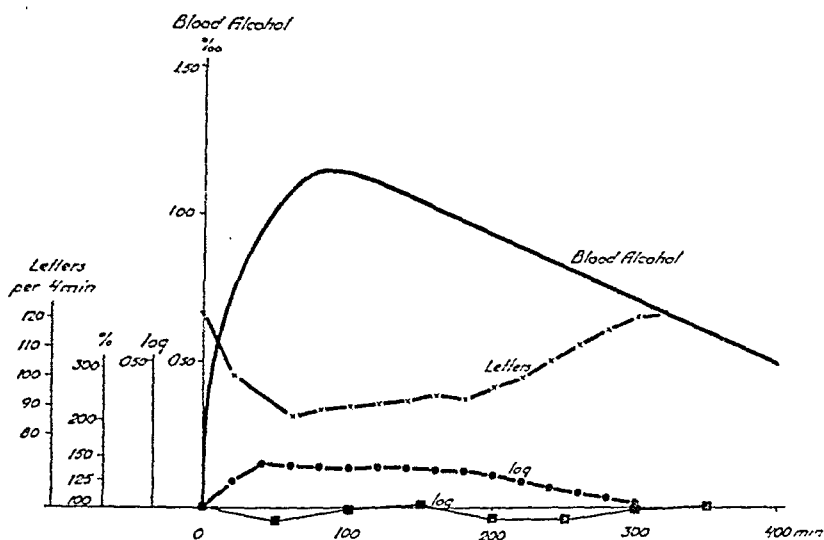
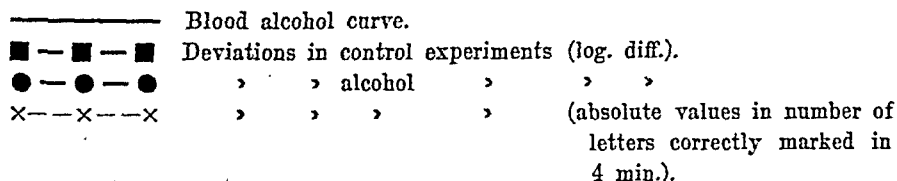


Fig. 17. Bourdon test (number of letters correctly marked).

Changes during alcohol intoxication. Mean of 17 experiments.



a circle was marked by a spiral or a large point, the markings were sometimes placed beside of the letters to be marked etc. (fig. 16).

The deviations from the normal were evident in cases of intense intoxication, a diminution of both the number of letters correctly marked and of the total read. The typical errors were in their order of frequency: 1) omitted letters, 2) the marking falling at the side of the letter, 3) incorrect markings.

Two different types of alcohol intoxication are illustrated in fig. 16. I being before alcohol, and II after.

In fig. 16 A the number of letters correctly marked (BC) diminished from 116 to 23, and the total number of letters read (BT) from 127 to 49 at a blood alcohol concentration of 1.88 ‰.

In the other case, another subject, the difference in quantitative performance was less marked: BC diminishing from 153 to 145 and BT from 165 to 161, but the atactic handwriting was more evident; the blood alcohol concentration was 0.88 ‰.

In Figs. 17 (BC) and 18 (BT) the average curves of experiments on 17 subjects were given, plotted against time in absolute values

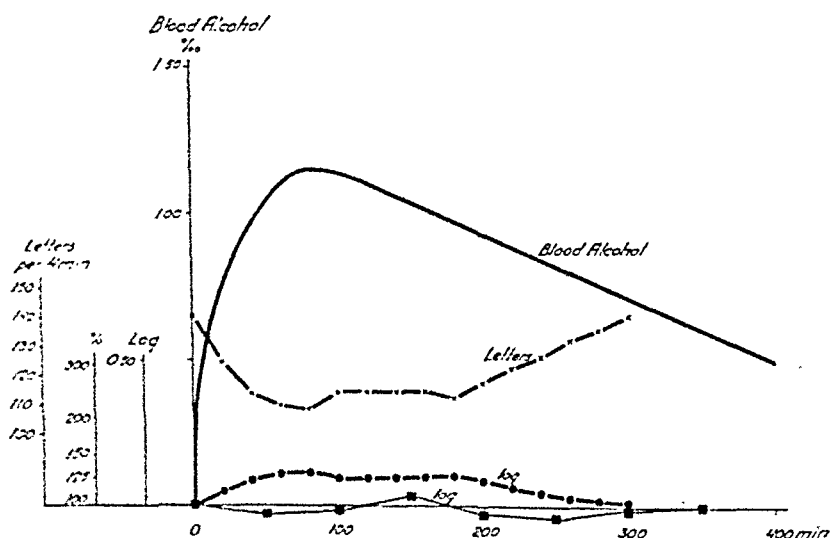


Fig. 18. Bourdon test (total number of letters marked).

— Blood alcohol curve.  
 ■ — ■ — ■ Deviations in control experiments (log. diff.).  
 ● — ● — ● , , alcohol , , ,  
 x — x — x , , , , (absolute values in total number  
 of letters read in 4 min.).

as well as in log differences. The absolute values followed a course opposite to that of the blood alcohol curve. The log differences were plotted with changed sign (+ in stead of —, cf. p. 16) to show the impairment in function.

The impairment of the results increased with increasing blood alcohol, and often showed a maximum before the maximum blood alcohol was reached. Then a rather horizontal course followed for one or two hours, in spite of the blood alcohol decreasing. After

that the log differences decreased with the falling part of blood alcohol and reached normal values before the blood alcohol had returned to normal.

The more or less horizontal part of the curve will be due to factors other than the influence of alcohol as it proceeds independently of the increase or decrease in the blood alcohol curve. One reason for this is the same mechanism of compensation which was observed in the subtraction test. Another reason will be the greater effect of alcohol during its increasing phase than during the falling. The taking of alcohol thus immediately impairs the attention and concentration of a subject. Then the compensating mechanism sets in, by means of an increased will-power or mental strain, which results in an increased concentration. This counteracts the influence of alcohol, and the effect measured by the number of the letters marked is the resultant of the action of alcohol and the amount of will-power mobilized. With the fall of blood alcohol the performance improves.

Another factor which also counteracts the effect of alcohol is the influence of practice. With the technique adopted the influence of practice was rather negligible (fig. 17 and 18 ■—■) but may have accounted for part of the horizontal course, as it plays a greater rôle during the first tests than later in the day, where fatigue comes in.

The log differences, finally, were plotted against the blood alcohol concentration, during the decreasing phase of the alcohol curve, which is illustrated in figs. 27 and 28 (●—●). The appearance and disappearance thresholds for the symptoms are given in table 6, cols. 1—3.

For both BC and BT the appearance thresholds are lower than the disappearance ones. The explanation for this, which also applies to the subtraction test, where the same phenomenon was observed, is that some kind of compensation sets in after the alcohol effect has begun. This causes the symptoms to return to normal earlier, *i. e.* at a higher blood alcohol level, than would have been the case, had no compensation taken place.

The thresholds were of the same order of magnitude as for the subtraction test, *i. e.* higher than for sensory and motor functions.

The slopes of the regression lines were not very steep, and the degree of intoxication at *e. g.* a blood alcohol concentration of 1 ‰ only 139 and 126 ‰ (BC and BT resp.).

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## Conclusions.

### Methods.

The following principal conditions that will have to be fulfilled so that a method or test may be suitable for following the influence of alcohol on a function, are:

1) The criteria of the alcohol effect as tested by the method should be constant, and should preferably leave no room for subjective judgment; if this factor cannot be ruled out, the method must permit of measuring its magnitude and bearing upon the results.

2) The variability of the method must be slight as compared to the changes which occur during alcohol ingestion.

3) If the test is to be applied for practical purposes, and no basal values are available, the variability between individuals should be slight as compared with the departures from normal due to alcohol.

4) The method must be "sensitive" in order to react on slight degrees of intoxication. The word "sensitive" can be interpreted in four different manners at least, as far as methods are concerned to reveal alcohol intoxication:

a) A slight variability under normal conditions.

b) Significant departures from normal at low alcohol concentrations, which correspond to a low appearance threshold.

c) A steep slope of the line of regression between log symptoms and blood alcohol, indicating a rapidly increasing degree of intoxication with slight changes in blood alcohol.

d) A slight variability after alcohol intoxication in relation to the slope of the regression line, giving highly significant departures from normal already at low degrees of intoxication.

The methods used were evaluated with these conditions in mind, and a survey of variability under normal circumstances is given in table 5.

*Variability of a Single Determination.* The tests showed a variation of a single determination of 5.4—20.3 % (table 5, col. 5), when ascertained from double determinations, carried out with an interval of 30—40 minutes.

*During the day.* If calculated from a series of experiments during a whole day (table 5, col. 8) the variation rose, its range being 8.5—72.2 %. If the figure for the modified Romberg is omitted the variation will be 8.5—26.0 %.

The greater variability during a whole day will primarily be connected with the influence of practice and fatigue which may play a rôle in these circumstances (9 determinations in 6—7 hours). These two factors were shown to counteract each other with regard to the *mean* for all functions except the subtraction test (p. 64), as the absolute values of the functions did not change. On the other hand, the increase of *variability* will be due to fatigue; the great difference between the variability of a single determination and that during the day in the modified Romberg position is no doubt due to this function being highly influenced by fatigue.

It is striking that so widely differing methods as tests on sensory, motor and psychological functions, with one exception, show a variability which is of the same order of magnitude. CROZIER, WOLF and ZERRAHN-WOLF (1937 b) stated the interesting fact that the values of the percentage error of a function are of about the same order of magnitude in very different sensory effects: in measurements of auditory intensity, discrimination in judgements of differences in lifted weights and in tests on flicker visual functions with the human eye. CROZIER and HOLWAY (1940 a) found the percentage standard deviation of the flicker response curve to be 10—15 %, and to remain constant in spite of variations in flicker frequency or in brightness for fusion. They consider the percentage standard deviation, which applies also to the logarithmic variance, to be constant and a property of the reacting organism; not only within the range of error for one and the same function at a given level in different individuals, but also for different functions, and finally in one and the same individual under widely differing conditions.

My findings are in full accordance with these arguments: the mean variability ( $\sigma$ ) of the functions studied was under normal conditions 9.0 %. But the analogy might be extended. A prelim-

Table  
*Basal Values and Variability of*

Test	Basal Values			Between Single Determinations		
	Number of Individuals	Log	Abs. Value	Log	%	Abs. Value
	1	2	3	4	5	6
Flicker test . . . .	29	$2.681 \pm 0.0777$		0.0355	8.82	
Corneal test . . . .	18	$1.476 \pm 0.0458$	29.9 mm Hg	0.0333	7.66	2.29
Stand. Stand. (ord. Rb)	39	$2.479 \pm 0.0242$	301.3 mm <sup>2</sup>	0.0261	6.00	18.07
"    (mod. Rb)	39	$2.923 \pm 0.0842$	837.5 mm <sup>2</sup>	0.0882	20.29	169
Finger-Finger test .	37	$2.300 \pm 0.0159$	199.5 mm <sup>2</sup>	0.0356	8.19	16.35
Subtraction test . .	37	$1.635 - 2 \pm 0.0266$	0.431 min	—	—	—
Bourdon test (BC) .	29	$2.095 \pm 0.0139$	124.5 letters	0.0235	5.41	6.74
"    (BT) .	29	$2.146 \pm 0.0125$	140.0 "	0.0302	6.95	9.73

inary calculation of the percentage error under alcohol intoxication was made with two methods: the finger-finger test and the subtraction test (pp. 60, 65).

The differences between double trials, in absolute values as well as in logarithms, were plotted against the mean of the two trials, expressed in the same units. The absolute differences showed an increase with increasing mean, whereas the logarithmic differences were approximately constant.

As the mean rose with increasing blood alcohol concentration, the findings imply that the absolute value of the standard deviation increased with increasing blood alcohol: the scatter of the performance augmented. The logarithmic variance remained constant, which implies that the percentage scatter did not alter with the changes in blood alcohol. Finally, the logarithmic variance was of the same magnitude as that under normal conditions, which is another reason for using logarithms and logarithmic variances for the evaluation of tests.

*Variability from day to day.* The variability from day to day showed no significant changes from the variations during the day. The results were not consistent, but suggest a slight increase which

## Tests under Normal Conditions.

V a r i a b i l i t y ( $\sigma$ )

During the Day			From Day to Day			Between Individuals		
Log.	%	Abs. Value	Log	%	Abs. Value	Log	%	Abs. Value
7	8	9	10	11	12	13	14	15
0.05698	13.10		0.0768	17.68		0.4184	96.34	
0.04276	9.84	2.94	0.0343	7.90	2.37	0.1943	44.74	13.38
0.1127	25.95	78.19	0.0987	22.72	68.47	0.1511	34.79	104.82
0.3138	72.25	605.09	0.336	77.36	646.87	0.5260	121.12	—
0.06694	15.41	30.74	0.0902	20.77	41.45	0.0968	22.29	44.47
0.05260	12.11	0.052	0.0808	18.60	0.080	0.1620	37.30	0.161
0.03854	8.87	11.04	0.0346	7.97	9.92	0.0752	17.33	21.58
0.03713	8.54	11.96	0.0260	5.93	8.37	0.0658	15.15	21.21

supports the procedure of relating a value found to the basal value of the day.

*Between individuals.* The variability between individuals showed certain interesting features. The Bourdon and the finger-finger test had the least variations, and the modified Romberg the greatest. As the variability was calculated from the logarithmic variance, the largest variability, that of modified Romberg, could not be transformed to absolute values (p. 19). For all tests beside the modified ROMBERG the variation *between* individuals exceeded the variation *within*. This was proved by analysis of variance for the 12 control experiments, which were performed on 12 different subjects, and the variation within based on 8 values.

## Alcohol Experiments.

A survey of the results of the methods used after alcohol ingestion is given in table 6, where the data of average curves are presented, and in table 12, where means of individual curves are given.

The plotting of the degree of disturbance against time shows the approximate coincidence with the blood alcohol curve. This fact



Table 6.  
*Thresholds, Slopes, and Degree of Disturbance of Tests after Alcohol Ingestion in 17 Normal Individuals.*

T e s t	Threshold in promille		Difference (2-1)	Variability	Slope		Degree of Disturbance Percentage (Normal = 100)	
	During Rising	During Decreasing			(From Con- trol Exps.)	Log	"σ-units"	At a Concen- tration of Alcohol in the Blood of 1 ‰
1	2	3	4	5	6	7	8	
Flicker Frequency . .	0.54	0.529	- 0.01	0.05698	0.3596	6.32	151	328
Corneal Sensitivity . .	0.31	0.363	+ 0.05	0.04276	0.3948	9.23	178	248
Stand. Stead. (ord. Rb)	0.65	0.659	+ 0.01	0.1127	1.9254	17.08	490	8,422
,   (mod. Rb)	0.41	0.623	+ 0.21	0.8188	3.0696	9.78	1623	117,400
Finger-Finger test . .	0.39	0.556	+ 0.17	0.06694	0.5208	7.77	174	331
Subtraction test . . .	0.49	0.748	+ 0.26	0.05260	0.2755	5.24	121	189
Bourdon test (BC) . .	0.48	0.702	+ 0.22	0.03854	0.4485	11.64	139	281
,   (BT) . .	0.40	0.722	+ 0.32	0.03718	0.3719	10.92	126	235

which has been proved in man by a great number of investigations on one or another function (VERNON 1919, MELLANBY 1920, HAMMARSTEN and LILJESTRAND 1922, MILES 1924, GRAF 1933, BAHNSEN and VEDEL-PETERSEN 1934, SCHMIDT 1934, WIERENGA 1935, ELBEL 1937, DANGER 1939, NEWMAN and FLETCHER 1940) strongly indicates a causal connection between blood alcohol and the degree of intoxication

Only few attempts are made to correlate quantitatively the degree of disturbance and the blood alcohol concentration. VERNON (1919) reported a rectilinear relationship to exist between the effect, as measured by his "target" method, and blood alcohol. GRAF (1933) measured the degree of disturbance with psychotechnical methods as percentage *increase* from normal, which was termed as 0 %, and tentatively fitted the data of one case by a second degree curve. This would imply that the degree of disturbance grew proportionately to the square of blood alcohol. In a second case the percentage *decrease* from normal was plotted against blood alcohol and treated in the same manner. He did not, however, prove that no other curve would fit his data equally well or better.

STRAUB (1938) reported similar experiments on 16 subjects and interpreted his results as confirming the findings of GRAF: the percentage of errors to increase proportionately to the square of alcohol. Unfortunately his original values were not published.

My findings suggest an approximate rectilinear relationship between the *logarithm* of the symptom, or the logarithmic difference which gives the same curve, and the blood alcohol concentration during the decreasing part of the blood alcohol curve.

The relationship during the decreasing part, when absorption is completed, favours the assumption that "tissue" alcohol, if in the brain or somewhere else in the central nervous system, causes the disorder rather than blood alcohol. The tissue alcohol lags behind the blood alcohol, which is due to the time factor of the diffusion processes (GOLDBERG 1937 a, TEORELL 1937 a, b, BERGGREN and GOLDBERG 1940), and therefore the strict correlation between blood and tissue alcohol is not withheld before absorption and distribution are completed. — The straight relationship was not found until 140—160 minutes after ingestion, thus approximately after completed distribution.

The same relationship was also found during the increasing

part of the curve by graphical computation. The facts suggest the relation to be part of a general mechanism as the two relation curves, the increasing and the decreasing one, coincided approximately as to slope, but differed as to threshold value.

As to the possible theoretical bearing of the correlation found, the following can be said:

As far as the flicker test is concerned, the correlation between log intensity and blood alcohol is in full accordance with other findings on that function (GRANIT *et al.* 1930, CROZIER *et al.* 1937—41, McFARLAND and HALPERIN 1940). As to the other functions tested it is a striking coincidence that all functions approximately showed the same correlation between the logarithm of a function and the blood alcohol. This is not in accordance with the Weber-Fechner law of the psycho-physical correlation, which implies the correlation between the logarithm of the undetermined variable and the absolute value of the determined variable, in this case the function tested.

On the other hand, other curves, too, have been discussed to fit experimental data: the probability curve and the curve of quantum discrimination, etc. No attempts have been made, however, to correlate the data presented with any of these theories. For practical purposes the rectilinear relationship found between log function and blood alcohol yielded satisfactory result as to agreement with experimental evidence. The curve allowed for computing the appearance and disappearance thresholds, its slope was an expression of the rate at which alcohol influences the interaction of the central nervous system, and it permitted within the ranges of error the calculation of the possible degree of intoxication at a given blood alcohol percentage.

*Appearance threshold.* The absolute values of the average appearance thresholds (table 6, column 1) were only approximate, this relationship being evaluated graphically and based on only few values (p. 22). Their range was 0.31—0.65 ‰, and no significance will be attributed to possible differences. The mean values for the three groups of functions were of the same order of magnitude. The sensory functions had a mean appearance threshold of 0.43 ‰, the motor functions of 0.48 ‰, and the psychological functions of 0.46 ‰, the differences not being significant.

*Disappearance threshold.* The average values of the disappearance threshold (table 6, col. 2) indicate a certain difference between

the groups, the sensory functions having the least (0.46 ‰), and the psychological the highest threshold (0.72 ‰) in relation to motor functions (0.61 ‰). That means that the disorders of the sensory functions lasted longest, and those of the psychological functions disappeared first.

When comparing the appearance and the disappearance thresholds (table 6, column 3), two things were visible: firstly, that the disappearance thresholds as a whole showed somewhat higher values than the appearance ones; and secondly, that this difference varies from function to function in a regular manner. For the sensory functions the thresholds coincided, the mean difference being + 0.02 ‰; for the motor functions the mean difference was + 0.13 ‰, and for the psychological functions + 0.27 ‰. One of the causes of this difference in appearance and disappearance thresholds will no doubt be *compensation*.

HOLMES (1917, 1939) points to the fact that symptoms of acute cerebellar injury (gun-shot etc.) gradually decrease in intensity and may in time disappear. This fact has been explained by compensation by other parts of the nervous system. But even in the acute stages of disease the disturbances may be to some extent reduced by training and by concentrated effort: "the patient learns to compensate". As learning is predominantly a function of the higher levels of the nervous system, the cortex, and especially those parts of it concerned with the integration of voluntary movements, probably play a large part in compensation (HOLMES 1939).

Applying my findings to this theory of compensation would imply that no compensation exists for the sensory functions, as far as they are involuntary. A certain amount of compensation was found for the motor functions, and a considerable for the psychological functions. This also explains why the psychological functions were most influenced by practice.

The same phenomenon was indicated by another fact: When plotted against time, the course of intoxication followed that of blood alcohol. A closer study revealed that the maxima of disturbance of the two sensory functions showed a tendency to occur later than the blood alcohol maximum (figs. 6 and 8). The maxima of the degree of disorder in the motor functions coincided with the blood alcohol maximum (figs. 10, 11, 14), whereas those of the psychological functions appeared decidedly earlier than that of blood alcohol (figs. 15, 17, 18). These facts likewise indicate a

difference in the rate of disappearance, the psychological functions showing the earliest improvement, and the sensory the last.

*Slope.* The slope of the line of regression (table 6, column 5) indicates the rate at which the alcohol influences a function. It is evident from table 6, that the motor functions are influenced at the highest rate: a slight increase in blood alcohol over the threshold value is followed by an enormous increase in degree of disturbance; an increase of 1 ‰ e. g. for the ord. Romberg test by 8422 % (table 6, col. 8).

One of the conditions for a good test was that the increase due to alcohol, *i. e.* the slope of the regression curve, must differ significantly from the experimental error. The slopes were related to their experimental error, and for simplicity expressed as products of the logarithmic standard deviation ( $\sigma_d$ ) during a day (table 6, col. 4, which is identical with table 5, col. 7). The figures give an approximate value of the slope in " $\sigma_d$ -units". They all show high values, and the motor functions the greatest, in spite of their larger variability, indicating these tests to be impaired to a high degree and suited for significant evaluation. The subtraction test was the least suitable, this being due to practice not being ruled out.

A graphical evaluation of the slopes of the *increasing* curves gave no significant change from the decreasing ones.

MELLANBY (1919, 1920) and MILES (1924) claimed that a given height on the rising side of the alcohol curve is accompanied by a much stronger measurable effect than is the same concentration level on the declining side; the same has been reported by other observers (MIRSKY *et al.* 1941, LINDE pers. comm.). My findings support these principal suggestions as to the difference between increasing and decreasing blood alcohol, and elucidate that this phenomenon did not exist for the sensory functions tested, but was seen for motor, and marked with psychological functions. One of its characteristic features was the lower appearance threshold for the symptoms. As the relationship was rectilinear between logarithm of symptom and blood alcohol, this implies that a given increase in blood alcohol over the threshold value has a much stronger effect on the rising side; equality of logarithmic slopes means widely different slopes of the absolute curve, if they are entered on differing levels.

*Variability of thresholds between individuals.* The graphical computation of the individual disappearance thresholds was given

in table 12, cols. 4—6. It was tentatively computed that the variation between individuals was about 30 %, and was significantly greater than the error of a single threshold. Moreover, by graphical computation according to BLISS (1938) it was proved that the thresholds were approximately normally distributed.

The disappearance thresholds are an expression for the tolerance to alcohol of an individual. Thus these results suggest tolerance to alcohol to be normally distributed, and the differences between individuals to be larger than the experimental error, which means that tolerance *varies* at random from individual to individual within a homogenous group of individuals.

Summarizing the facts about the variability of the methods used and their application for testing alcohol intoxication, it may be stated that the sensory functions were influenced at the lowest, and the psychological at the highest blood alcohol level. As to the magnitude of departure from normal, the *motor* functions were most influenced, both absolutely, as indicated by the values in table 6, column 7, at a level of 1 ‰, and relatively: they showed the highest slope and the greatest departure from normal at a given increase in blood alcohol concentration (table 6, col. 8).

As to the question about practical applicability, all tests yielded results if related to a basal value. Can a basal value not be obtained, quantitative records of the *motor* functions, primarily the ordinary Romberg position, show departures from normal which widely exceed the variation between individuals. If the values of the slopes (table 6, col. 6) are expressed in " $\sigma_i$ -units", and the inter-individual variations (table 5, column 13) are taken as  $\sigma_i$ , the different tests, in the sequence flicker, corneal, ordinary Romberg, modified Romberg, finger-finger, subtraction, Bourdon (correct) and (total), give 0.861, 2.03, 12.72, 5.83, 5.37, 1.70, 5.96 and 5.65 " $\sigma_i$ -units". Thus flicker frequency, corneal sensibility, and subtraction test are not suitable *absolute* tests on the degree of alcohol intoxication.

On the other hand, the influence of practice after a few preliminary trials and of variations from day to day is negligible (cf. the difference between cols. 5 and 8 in table 5); thus a basal value can be procured on another occasion, the next day etc., which improves the suitability of the tests for practical purposes most considerably.

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## PART II.

### The Influence of Food Intake on Alcohol Tolerance.

A considerable number of investigations on animals and in man have established that the concentration of alcohol in the blood has a different course if the alcohol is taken on an empty stomach or with food. In the latter case that part of the curve which is due to absorption is prolonged, and its maximum is lower and appears later than in fasting. The diminution in height can correspond to as much as 10—20 % of the total amount (WIDMARK 1916). Further the distribution phase is no longer characterized by its steep decrease, but it becomes smoothed out and can follow a horizontal course for a shorter or longer period of time. Finally, the rate of decrease during the post-absorptive part of the curve differs widely from case to case.

MELLANBY (1919) in experiments on dogs attributed these changes to the delay of absorption, which was most influenced when the food taken contained milk and fat. HAGGARD, GREENBERG and LOLLI (1941) also found that milk had the greatest effect in man, but butterfat exercised the least influence on absorption.

WIDMARK (1933, 1934) in experiments on dogs and in man stated that not only absorption was delayed, but that in some cases the blood alcohol concentration never reached those values which were found when alcohol was given on an empty stomach, thus a lowering of the whole alcohol level under food conditions. On the basis of experiments with NEYMARK (1936 a), WIDMARK claims that this disappearance of alcohol is due to part of the alcohol never being absorbed as such. He ascribed this to the alcohol being bound to some constituent parts of the food as proteins and amino acids, *e. g.* glycerol, by means of an ester-formation.

His observations were criticized by HAGGARD and GREENBERG (1940) and EGGLETON (1940 a, b). The first mentioned authors concluded from experiments on rats that glycol induced a prolonged retention of alcohol in the stomach, and a correspondingly retarded absorption, enabling the alcohol to be oxidized as rapidly as it was absorbed. The concentration in the blood was thus kept down to an extremely low value, and no theory of ester-formation was necessary to explain the lowering of the blood alcohol level. Their results cannot however be applied to man. The mucous membrane of the stomach of the rat differs in its anatomy from that of man, which involves a different mechanism of absorption. In the rat only a slight percentage of the alcohol ingested is absorbed in the stomach and only concentrations up to 6 % (DYBING and RASMUSSEN 1940), whereas in man 20—50 % of the alcohol ingested is absorbed already in the stomach and up to a concentration of 30—50 %.

EGGLETON (1940 a and b) reported that in cats the disappearance of alcohol when ingested with food besides being due to a possible ester-formation according to WIDMARK (1934), was also due to the specific-dynamic effect of amino acids, *e. g.* alanin, on the metabolism of alcohol. The alcohol was said to be combusted at a higher rate during absorption than at subsequent periods of alcohol metabolism. This would induce deviations from the ordinary fasting alcohol curve and appear as a loss of alcohol. As EGGLETON found an exponential curve to fit the blood alcohol data of cat during fasting, this makes her conclusions not applicable to the results in man, where a rectilinear curve dominates (p. 23).

Concerning the degree of depression of the blood alcohol curve MILES (1922 a) claims this to be due to the diluting effect of the contents of the stomach, *e. g.* food, on the alcohol ingested, weak alcoholic solutions being absorbed at a slower rate than more concentrated ones. This would be in full accordance with the diffusion theory of absorption and is supported by the observations of BERGGREN and GOLDBERG (1940) who in experiments on animals and human beings found the absorption of alcohol in the stomach to follow the laws of diffusion.

The experiments of ELBEL and LIECK (1936) showed the same tendency. They found the blood alcohol level to vary with the quantity of food ingested. Their results thus do not confirm the observations of JUNGMICHEL (1933), which implied the caloric intake to determine the blood alcohol level.



Experiments on the degree of intoxication after alcohol taken with a meal all show an obvious decrease in the intensity of the alcohol effect produced, as compared with the action of alcohol when taken on an empty stomach (WEISSENFELD 1898, VOGT 1910, VERNON 1919, McDOUGALL and SMITH 1920, HAMMARSTEN and LILJESTRAND 1922, MILES 1922, 1924, BLOTNER 1939, DANGER 1939, and HAGGARD, GREENBERG and LOLLI 1941).

These facts are easily interpreted as the consequence of the lowered blood alcohol curve in these cases, which has been emphasized by HAMMARSTEN and LILJESTRAND, MILES, and BLOTNER among others. No investigations, however, have been made on the possible relationship between the decrease in effect and that of the blood alcohol level. The following experiments are intended to give an orientation on this problem, approached by means of quantitative measurements of a series of different functions according to the methods already described in Part I.

### Experiments.

The experiments on the action of food on blood alcohol and on the degree of intoxication were divided into two parts.

In a preliminary series of seven experiments each subject served only for one experiment, and the alcohol was taken either with a *slight* meal consisting of one slice of bread and butter or a bit of sponge-cake, or one and a half hours *after* a meal.

In the final series, comprising twelve experiments in all, the alcohol was taken at the end of a substantial meal (p. 12). Six subjects took part in this series, each serving for two experiments, one without and one with food. The technique used was that already described in Part I.

#### The Blood Alcohol Curve after Food.

The influence on the blood alcohol curve of a meal, taken with the alcohol, is illustrated in fig. 19 A, B, C. In each figure the fully drawn curves correspond to the blood alcohol level when the alcohol was taken on an empty stomach and the dotted curves correspond to the alcohol level after the taking of the same quantity of alcohol with a meal.

The "food curve" in fig. 19 A shows a large depression in relation to the fasting curve with a low maximum and a slowly decreasing part. This finding must be interpreted as the result of a great delay in absorption both as concerns rate and time, absorption continuing for 4—6 hours.

In the case, illustrated in fig. 19 B, a plateau formation was maintained for 2—3 hours after a delayed absorption period. The horizontal course was then followed by a rather rapid fall. The horizontal course of the curve must imply that absorption and

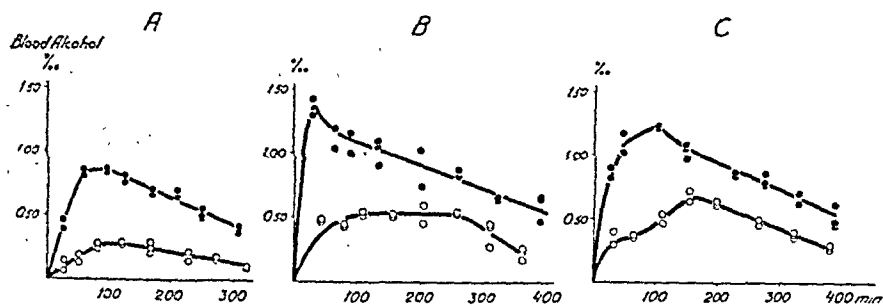


Fig. 19. The influence of a meal on the blood alcohol curve.

- — ● Alcohol taken on an empty stomach.  
 ○ — ○ „ „ „ with a meal.  
 (Explanation see text.)

distribution on the one hand and combustion on the other balance each other. When absorption was completed, distribution was still maintained which caused the rapid fall of the post-absorptive phase.

Finally, in fig. 19 C, a case is illustrated where the post-absorptive period had a parallel course to that of the fasting curve. The explanation will be that absorption in this case was delayed to such an extent that the maximum appeared later and the normal over-shooting of alcohol was prevented. The distribution phase was not reflected in the blood alcohol curve as under fasting conditions and the rate of disappearance of alcohol from the blood stream during the post-absorptive period was the same as in fasting.

All of these cases together with the experiments of the preliminary series support the assumption of WIDMARK of the loss of alcohol: In no case did the level of the "food" blood alcohol curve reach the values of the "fasting" curve.

WIDMARK (1932) and NEYMARK and WIDMARK (1936 b) pointed clearly to the fact that the factors  $\beta$  and  $r$  are only to be calculated when alcohol is taken on an empty stomach.

The calculation of the factor  $\beta$  or  $r$  respectively from the decreasing part of the curve under food conditions is very difficult in most cases because of the irregular course of the alcohol curve. An attempt was made to obtain an approximate value of  $\beta$ . These values are given in table 7, and were generally computed with the blood alcohol value *after* the maximum as first value for the calculation. They all showed a tendency to be lower than under fasting conditions.

A computation of  $r$  under the same circumstances showed this factor to be decidedly higher than in fasting, and not only due to the lowering of  $\beta$  (NEYMARK and WIDMARK 1936 b), but also due to the fact that the whole curve was depressed in relation to the fasting curve. (Fig. 19 C).

The possible loss of alcohol due to ingestion of food can be obtained in different ways, either from the change in  $c_0$  or by means of a so-called "backward calculation" according to WIDMARK (1932). Both ways gave a possible loss of alcohol of about 40 %. Similar figures were found by SCHMIDT (1937) and ELBEL (1937).

### Alcohol Tolerance after Food.

The degree of intoxication following the taking of alcohol with a meal was ascertained on six subjects. On each subject two experiments were made, one when the alcohol was taken on an empty stomach, and the other when it was taken with food within the last ten minutes of the meal (p. 12). The experiments were arranged as has been previously described (p. 11). Thus eight different functions were studied during six to eight hours and the degree of intoxication referred to the prevailing blood alcohol concentration.

The principal findings were exactly the same in all functions studied, and are therefore not treated separately. A typical result is illustrated in fig. 20, which shows the changes in brightness for fusion after alcohol ingestion in fasting and after a meal.

In fig. 20 (upper part) the changes were plotted against time. The course of log brightness followed the blood alcohol curve during fasting as well as after food, increased with the increasing

Table 7.  
*Comparison of Alcohol Factors after Ingestion of Alcohol on an Empty Stomach and after Food.*

Case Nr	Rate of Disappearance of Alcohol from the Blood ( $\beta$ )			Distribution Ratio ( $\gamma$ )			Combustion mg alc / kg / hour ( $\beta \times \gamma \times 60$ )			Alcohol Loss				
	Fasting	Food	Difference (2-1)	Fasting	Food	Differ- ence (5-4)	Fasting	Food	Difference (8-7)	c <sub>o</sub> (°/∞)		Difference		
										Fasting	Food	(11-10) °/∞	Percentage (12/10 × 100)	
1	2	3	4	5	6	7	8	9	10	11	12	13		
I	0.00218	0.00115	- 0.00103	0.660	1.524	+ 0.864	86.3	105.1	+ 18.8	1.061	0.460	- 0.601	- 56.6	
II	0.00242	0.00160	- 0.00082	0.654	0.952	+ 0.288	96.6	91.3	- 5.3	1.506	1.050	- 0.456	- 30.3	
III	0.00200	0.00200	± 0	0.800	0.877	+ 0.077	95.8	105.2	+ 9.4	1.230	1.140	- 0.090	- 73.2	
IV	0.00180	0.00196	+ 0.00016	0.788	1.077	+ 0.294	84.6	112.6	+ 28.0	1.277	1.020	- 0.257	- 20.1	
V	0.00214	0.00182	- 0.00032	0.731	1.038	+ 0.307	93.9	113.2	+ 19.3	1.368	0.968	- 0.405	- 29.6	
VI	0.00234	0.00130	- 0.00104	0.654	1.005	+ 0.351	94.0	78.1	- 13.9	1.532	0.995	- 0.537	- 35.1	
Mean			- 0.00051 %			+ 0.363			+ 9.38 mg/kg/hr				- 40.8 %	

part of the curve, its maximum appearing at approximately the same time as the blood alcohol maximum, decreased with the decreasing part of the alcohol curve, and reached its basal value before the blood alcohol had returned to normal. The degree of intoxication was, however, decidedly lower after food (O—O).

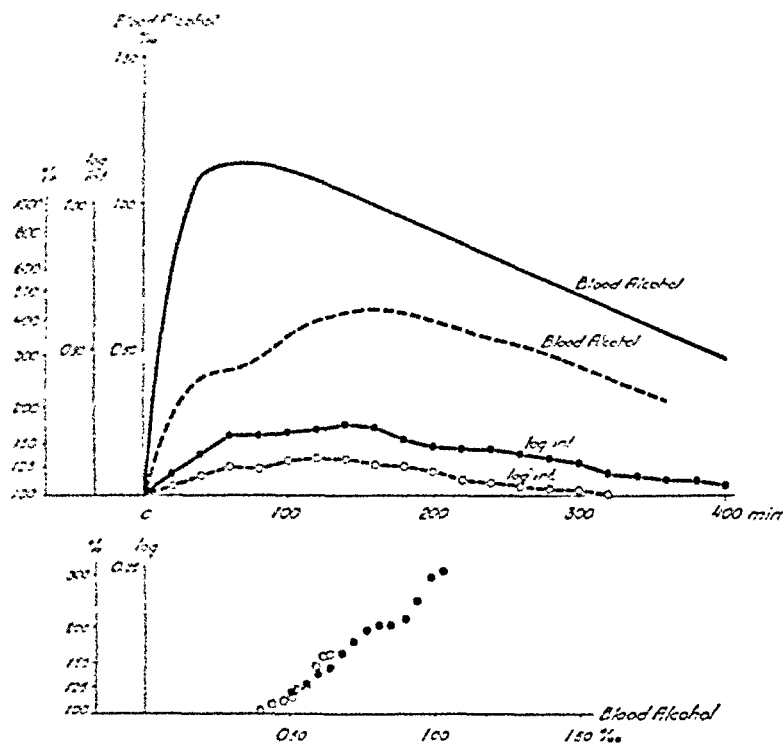


Fig. 20. The influence of a meal on the changes in brightness for fusion after alcohol ingestion. Mean of 6 experiments.

Log intensity plotted against time in the upper graph and against blood alcohol in the lower.

- Blood alcohol (fasting).
- - - - - , , (after a meal).
- — ● Log intensity (fasting).
- — ○ , , (after a meal).

Referring the degree of intoxication to blood alcohol (fig. 20, lower part) shows two facts. The first is that the rectilinear relationship during the decreasing part of the alcohol curve between the log brightness and the concentration of blood alcohol (p. 34) was maintained also under food conditions. The second that the relationship, representing the correlation

between log symptoms and blood alcohol in the food experiments coincided completely with that found in fasting.

A survey of the threshold values of the decreasing part of the disturbance curves and the slopes of these curves, when referred to blood alcohol, is given in table 8. No significant changes between the threshold values in fasting or after food were found, nor

Table 8.

*The Influence of a Meal on the Relationship between log Symptoms and Blood Alcohol Concentration.*

Disappearance Thresholds and Slopes of Regression Lines after Alcohol Taken on an Empty Stomach (E) and with a Meal (F). Mean of six Experiments.

T e s t	Disappearance Threshold (‰)			Slope (log units)		
	E	F	Diff. (F—E)	E	F	Diff. (F—E)
Flicker test . . . .	0.40	0.41	+ 0.01	0.34	0.40	+ 0.06
Corneal test. . . .	0.44	0.56	+ 0.12	0.50	0.49	— 0.01
Stand. Stead. (ord. Rb)	0.62	0.51	— 0.11	2.80	2.10	— 0.70
"    (mod. Rb)	0.50	0.58	+ 0.08	2.51	3.09	+ 0.58
Finger-Finger test .	0.69	0.50	— 0.19	0.94	0.64	— 0.30
Subtraction test . .	0.63	0.64	+ 0.01	—	—	—
Bourdon test (BC) .	0.65	0.57	— 0.08	0.61	0.98	+ 0.37
"    (BT) .	0.62	0.60	— 0.02	0.41	1.23	+ 0.82
Mean			— 0.022			+ 0.14

between the corresponding slopes. The variations were within the range of errors, and no significance can be attributed to possible differences between single functions.

The conclusions of these experiments are that the decrease in the degree of intoxication which followed the taking of alcohol with food, corresponded to the alterations in the blood alcohol level. The relationship between the symptoms and the concentration of alcohol in the blood ascertained during fasting conditions, was maintained within the range of error when alcohol was taken with food.

This suggests that the effect of a meal on the symptoms fol-

lowing alcohol ingestion is purely physical, due to the lowering effect of food on the concentration of alcohol in the blood. The effect is probably not due to any specific action of digestion products on the sensitivity of the central nervous system to the influence of alcohol. The experiments strongly support the assumption that in one and the same individual the blood alcohol level determines the degree of intoxication following the intake of alcohol.

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### PART III.

## The Influence of Habituation on Alcohol Tolerance.

### Experiments on Abstainers, Moderate and Heavy Drinkers.

*Habituation* to a drug in its pharmacological sense means the changed reaction of the organism to the prolonged use of it, generally in the direction of an increased tolerance. This implies less obvious symptoms after doses, which would cause marked disturbance in an unhabituated individual.

The phenomenon of *addiction* must not be confused with that of habituation. Addiction primarily means the inability of an individual to withdraw from the prolonged use of a drug in spite of, or maybe due to, its deleterious effect on the organism. If addiction is combined with habituation, the individual must augment the dose administered in order to produce the desired effect. Moreover, abstinence symptoms, *i. e.* disorders owing to the withdrawal of the drug, also form part of the conception of addiction. It has now been unquestionably proved that habituation and addiction to drugs does exist, *e. g.* to narcotics belonging to the morphine group. As to ethyl alcohol, opinions differ widely.

Experience suggests that people differ in their reaction to the intake of alcohol and that abstainers behave differently from those used to occasional or daily alcohol consumption.

The first question is whether any evidence exists for this empirical assumption, *i. e.*: Do there exist differences in tolerance to alcohol between individuals, and can these be attributed to their alcoholic habits, or can the differences be explained by the variations in tolerance, which are known to exist among a population?

Evidence of varying tolerance to alcohol is now accumulating, especially as the result of medico-legal examination after traffic



accidents. The differing criteria for diagnosis are one of the causes of the apparent divergence in tolerance (p. 46), but even after the elimination of that source of error there remains an evident difference in tolerance between single individuals, some people being influenced even at the concentration of alcohol in the blood of 0.5 ‰, corresponding to 0.3 to 0.4 g alc. per kg, others not until concentrations over 2 ‰, corresponding to 1.5 g alc. per kg.

Only two observers seem to have tried to analyse a *clinical* material of varying tolerance with regard to habituation to alcohol. JETTER (1938 a) examined 1,000 patients with acute alcoholism and correlated clinical criteria of intoxication and blood alcohol. The results were compared with similar observations on 20 abstinent individuals, who were given 1 to 2 cc alcohol per kg (JETTER 1938 b). At a blood alcohol concentration of 2 ‰, 83 % of the chronic alcoholics were clinically intoxicated as compared with 100 % of the occasional drinkers.

HASSELBALCH-LARSEN (1940) examined 164 cases of acute alcoholism, divided up into normals, psychopathics and chronic alcoholic drinkers. The diagnosis of alcohol intoxication was ascertained by means of a clinical examination, and divided into five degrees varying from "slightly influenced" to "inebriated"; the result was referred to the concentration of alcohol in the blood. This observer found that a difference with respect to tolerance existed between the three groups examined, the chronic alcoholic addicts being less, and the psychopathics slightly more influenced than normal individuals at one and the same concentration of alcohol in the blood.

In *laboratory* work, however, only single experiments have been carried out on the possible influence of habituation in man. NEUMANN (1899), in experiments on himself, reported that the psychological effects of alcohol gradually decreased with the prolonged use of alcohol, and parallel to the decrease of nitrogen elimination in the urine.

Using psycho-technical methods to measure the degree of disturbance after alcohol ingestion, VERNON (1919) found a decreased effect of alcohol on one individual used to taking large quantities of alcohol in comparison with an occasional drinker, and the same was reported by GRAF and FLAKE (1933).

The experiments of KÜRZ and KRAEPELIN (1901) failed to show any effect of habituation on the performance of an addition test etc. This was referred by the authors themselves to the short

period of habituation being only a fortnight. VOGT (1910), FRANK (1925) and RIEGEL (1925), in experiments on themselves, saw less effects of alcohol on performing a psychological and a psychomotor test; their results are, however, not fully reliable owing to the uncontrolled influence of qualitative changes in the performance of the tests, which lasted for 2—3 months.

Thus, evidence based on statistical evaluation of clinical examination favours the assumption that there does exist an increase in alcohol tolerance with the degree of habituation, which is not contradicted by the tentative experimental findings in man.

As to studies on the possible factors which might lie behind habituation, workers in this field have primarily searched for changes in the *blood alcohol* level to explain the lowering of the degree of intoxication.

In *man*, MILES (1922) and GRAF and FLAKE (1933) in experiments on each one habitual, found no change from the curves of normal individuals. BERNHARD and GOLDBERG (1935) followed the course of the blood alcohol curve after alcohol ingestion in 19 alcoholic addicts, and compared the results with similar experiments on a large number of normal individuals. They stated that practically no difference existed between normal individuals and alcoholics as far as the rate of disappearance of alcohol from the blood ( $\beta$ ) and the distribution ratio ( $r$ ) was concerned. As to the rate of absorption their results suggested a more rapid absorption in alcoholics than in normal individuals. They concluded from their experiments that habituation or addiction to alcohol in man is *not* explained by changes in the blood alcohol level. Their findings were confirmed by SCHMIDT (1937) and SIEGMANN (1938), who likewise found no difference between alcoholics and normal individuals with regard to the blood alcohol curve.

These results in man are strongly supported by similar findings in *animals*. LEVY (1935 a, b) and NEWMAN and LEHMAN (1938) in rats, FAURE and LOEWE (1923) and KEESER and OELKERS (1937) in rabbits, and VÖLTZ and DIETRICH (1915), BALTHAZARD and LARUE (1921), NEWMAN *et al.* (1936 a and b, 1937 a, b, 1938), and NEYMARK and WIDMARK (1938) in dogs found no changes in the rate of disappearance of alcohol from the blood after submitting their animals to the prolonged use of alcohol for periods varying from one month to two years. — There is no explanation offered for the diverging results of PRINGSHEIM (1908) in rats and of GETTLER and FREIREICH (1935) in dogs. Their results, inter-

already stated (JETTER 1938), HASSELBALCH-LARSEN 1940, only tentative studies by HANSEN (1925 b) and DANGER (1939) have been made in *man* to compare the degree of intoxication in habituals and normal individuals with blood alcohol. Their results suggest a changed relation, but are, however, rather inconsistent. The evidence from experiments in *animals*, on the other hand, has yielded conclusive results.

In an extensive study on rats LÉVY (1935 a, b) proved a real habituation to exist, as the dose of alcohol necessary to produce anesthesia increased by 28 % and the lethal dose by 10 % in rats given alcohol daily for 1 to 8 months, as compared with normals. The rate of combustion was not changed (1935 a). The concentration of alcohol in the central nervous system was the same in both groups (1935 b). On the basis of these facts LÉVY concluded that habituation was due to the lowered sensitivity to alcohol of the nerve cells.

NEWMAN and CARD (1937 a and b) and NEWMAN and LEHMAN (1938) proved this to be true also in the dog. A number of dogs were accustomed to the daily intake of alcohol for varying periods of time. Nine different stages of alcohol intoxication were established by means of criteria of neuro-muscular in-coordination and referred to the blood alcohol level. The animals acquired a certain degree of tolerance after being fed with 10 % alcohol for three months. The habituated animals showed a less degree of disturbance of neuro-muscular co-ordination at moderate concentrations of alcohol than the controls, in spite of identical blood alcohol curves; with anesthetic doses no difference was found. The authors attributed this to the habituated organism being productive of a *psychological* adjustment for the long continued presence of alcohol in the brain, and of learning to compensate for this lack of co-ordination and removal of inhibitions (NEWMAN and CARD 1937 a). After 7 months abstinence the same animals lost their increased tolerance and were as intoxicated as the controls; the blood alcohol curves still coincided (NEWMAN and CARD 1937 b). In order to establish whether the increased tolerance was due to changed nervous tissue permeability, increased cellular tolerance or psycho-motor compensatory mechanisms, analyses of the alcohol content of the brain of rats accustomed to alcohol for 5 months were carried out. The blood alcohol curves coincided for habituated and control animals, whereas the brain alcohol was somewhat higher, indicating an increased permeability of alcohol in

the habituated organism (NEWMAN and LEHMAN 1938). They concluded that acquired tolerance to alcohol is primarily a tissue tolerance, whereby the cells of the central nervous system acquire the ability to function more effectively at a given alcohol concentration than before habituation.

So far we can say that habituation seems not to be connected with changes in blood alcohol, but with a changed relationship between symptoms and the blood alcohol level. This assumption is tentatively suggested in man, and strongly supported by evidence on animals.

Other theories, too, on the nature of habituation to alcohol have been formed. With psychological tests referred to blood alcohol, SCHMIDT (1937) found no conclusive differences between individuals with a differing alcohol consumption. He states that pharmacologically an habituation will scarcely exist, and doubts the clinical conception, too, that an alcoholic really can imbibe large quantities of alcohol without being apparently intoxicated. The mechanism of the alcohol habituation in its clinical sense is referred by him to two factors. The first is that the consumption is *distributed* over a long period of time with no subsequent cumulation in comparison with the moderate drinker, who occasionally takes the amount within a shorter time interval. The second is that the habituated individual learns to *compensate* psychically the symptoms of intoxication, by speaking slowly to compensate a blurred speech, by walking cautiously to avoid disturbances of gait, etc.

The conception of a psychic compensation being a cause of habituation is adopted by several workers. VIEBEG (1938) states that the objective degree of disturbance is approximately the same in an habituated and an unhabituated individual; the subjective difference is due to the difference in self-control and personality.

ANDRESEN (1938) maintains that the degree of intoxication ascertained by clinical examination depends *i. a.* on "the ability of the examined subject to pull himself together" and the influence of alcohol; people accustomed to consuming large quantities of alcohol would more easily pass the tests, as they have trained their ability to accomplish their functions in a state of inebriation.

MUELLER (1938) also accepts the idea that the habituated accomplishes functions familiar to the drinker, even at high alcohol concentrations, but he performs special tests as badly as the un-

habituated. This is in agreement with HEISE (1934), who stated that alcohol intoxication primarily appears in actions that have not become a habit. Neither could NEWMAN and CARD (1937 a) in their experiments on habituated dogs completely rule out the factor of a compensatory mechanism.

Summing up the reference on habituation there is as main feature a certain probability for the existence of an habituation to alcohol in the pharmacological sense, even if this be doubted in man. As to the nature of this phenomenon, it must now be considered as established that no changes in absorption, distribution or combustion can explain the possible habituation. The factors to be counted with are clinical statements on an acquired ability of the habituated individual to compensate psychically functions familiar to him, and experimental evidence in animals of a tissue tolerance, *i. e.* a changed reaction of the cells of the central nervous system to alcohol due to habituation.

---

The aim of this work with regard to habituation will be to prove that such a phenomenon really exists in man, to elucidate its characteristic pattern, and to analyse the relative importance of some of its features. For this purpose a series of experiments was performed on three groups of test subjects of varying degree of habituation: abstainers, moderate drinkers, and heavy drinkers. This last group consisted only to a small extent of alcohol addicts (p. 13), as the problem of addiction (see definition p. 95) lies outside the scope of this theme.

The degree of habituation was already seen in the behaviour of the subjects. In this paper, which is intended to show the general trend of alcohol intoxication in relation to the degree of habituation, only average results with the functions tested will be presented. A closer analysis of clinical and psychological findings in the single individual will be reported in detail later.

## Experiments.

### Procedure and Technique.

It was first intended to keep the same dosage of alcohol throughout all three groups, and use one gram per kg as a standard dose. It proved sufficient to create measurable disorders of all functions tested in the group of moderate

drinkers, and did not as a rule cause serious disturbances, such as vomiting, collapse etc. It was, however, generally too much for the majority of the abstainers, who either refused or were not able to take as much as the whole dose on an empty stomach without vomiting or even collapsing. The dose of one gram per kg to a habitual drinker on the other hand often failed to produce any visible symptoms which could be used for quantitative measurement, or they disappeared so rapidly as to return to normal before the blood alcohol had passed to its decreasing phase, *i. e.* that part to be closer investigated. The standard dose had consequently to be abandoned.

The dose for the abstainers was restricted to 0.63—0.80 gram alcohol per kg, in one case one gram per kg was administered. The moderate drinkers were given 1.0—1.42 gram per kg, and the habitual drinkers generally received 1.2 gram alcohol per kg, in some cases as much as 1.35 g. The latter showed no difficulties whatever in consuming that quantity of alcohol, neither was any vomiting or acute illness observed in this group.

In *preliminary* experiments the blood alcohol curve was followed and a thorough clinical examination repeated at regular intervals. These blood alcohol curves are included in the average but not the results of the clinical examination.

In the *final* series 39 complete experiments in all were carried out on 10 abstainers, 17 moderate drinkers and 10 heavy drinkers, in accordance with the procedure stated (p. 11). Thus all six tests: flicker frequency, corneal sensitivity, standing steadiness, finger co-ordination, subtraction and the Bourdon test, were performed twice previous to the alcohol ingestion, and at least seven times after the taking of alcohol. On every one of the subjects the results of the tests are fully comparable with each other. Only average curves will be discussed. In those cases where several experiments were carried out on one and the same individual, the mean of all experiments was used for the average curve, each subject being included once in the average.

### The Blood Alcohol Curve.

The general course of the blood alcohol curves in the three groups was the same: an absorption phase, the more or less marked absorption peak, a diffusion phase and the rectilinear, post-absorptive combustion phase.

The maximum seemed to appear somewhat sooner among the group of habitual drinkers than in the others. This tendency to a more rapid absorption with an earlier occurring maximum and a higher absorption peak was suggested by BERNHARD and GOLDBERG (1935), FLEMING and STOTZ (1935), and SCHMIDT (1937) in man, and VÖLTZ and DIETRICH (1915) and FAURE and LOEWE (1923), and NEWMAN and CUTTING (1936 a) in animals. It might be due to a higher incidence of gastritis and achlorhydria in alcoholic addicts (VILLARET *et al.* 1936, JOFFE and JOLLIFFE 1937). Experiments of HANEBORG (1920) and BERGGREN and GOLDBERG (1940) on the absorption of alcohol from the stomach suggest absorption to be accelerated in the case of gastritis due to the increased vascularization. Achlorhydria, which implies a quicker emptying of the stomach, also increases the rate of absorption of alcohol, this being more quickly performed in the intestines.

The values of  $\beta$  are of the same magnitude for abstainers and moderate drinkers. In heavy drinkers a certain tendency to higher values was observed, the difference being significant (table 9). The difference is not great, about as much as the individual variation, and of the same magnitude as that shown for alcoholic addicts with clinical signs of alcoholic disorders (BERNHARD and GOLDBERG 1935).

The slight difference may depend on the rapid absorption in this group as compared with the abstainers and moderate drinkers, and related to the dosage, which was larger. It may be that under such circumstances the absorption is not completed in 120 minutes, even if the curve has visibly passed over into the apparent rectilinear part. These circumstances would tend to elevate the value found for  $\beta$ . FLEMING and STOLTS report similar findings (1935). Against this argument there is the fact that the values for the distribution factor  $r$  showed no differences between the groups. As  $r$  tends to be smaller with increasing  $\beta$ , which is due to the manner in which it has been calculated (NEYMARK and WIDMARK 1936 b), an increase of  $\beta$ , due to inadequacies in calculation, would imply a corresponding decrease in  $r$ . This favours the assumption that the increase in  $\beta$  found in heavy drinkers is not due to its being calculated before the completion of the distribution.

Another explanation will be, that  $\beta$  tends to a higher value with a higher dosage, *i. e.* the blood alcohol curve is not rectilinear. Several facts discredit this (p. 23).

Table 2.  
*Alcohol Factors in Abstainers, Moderate Drinkers and Heavy Drinkers.*

	Abstainers			Moderate Drinkers			Heavy Drinkers			Probability (P) that the groups coincide
	Num- ber of Cases	Alcohol Factors	Stand. Dev. $\sigma$	Num- ber of Cases	Alcohol Factors	Stand. Dev. $\sigma$	Num- ber of Cases	Alcohol Factors	Stand. Dev. $\sigma$	
Rate of Disappearance of Alcohol from the Blood ( $\beta$ )	9	0.00209 %/o	0.000231	16	0.0023 %/o	0.000312	14	0.00266 %/o	0.000271	$< 0.001$
Distribution Ratio ( $r$ ) . . . . .	9	0.703	0.0763	16	0.723	0.0417	14	0.656	0.0332	$> 0.2$
Alcohol Metabolism $\beta \times r \times 60$ mg/kg/hour . . .	9	87.9 mg/kg/hr	10.28	16	95.5 mg/kg/hr	7.71	14	109.6 mg/kg/hr	4.93	0.01—0.001
Maximum of Absorption (min) .	10	72.3 min	35.10	22	88.3	26.91	17	57.6	33.13	$< 0.001$



Thus it must be concluded that the fact of there seeming to be an apparent slight difference in  $\beta$  between heavy drinkers and normal ones remains so far unexplained, and should be more thoroughly investigated. No difference in the distribution factor  $r$  was found in the three groups. The metabolic rate showed a slight and significant increase ( $0.001 < P < 0.01$ ), due to its being the product of  $\beta$  and  $r$ .

## A. Sensory Functions.

### 1. Brightness for Fusion.

The difference in basal values between the three groups (table 10) was significant, and indicated that the heavy drinkers showed a somewhat less ability to discriminate as their criterion for fusion lay at a somewhat higher intensity (intensity being the inverse value of log filter needed for fusion). Whether the different composition of the groups with regard to education (p. 13) has a bearing on the slight difference, or whether the difference is due to the possible effect of alcohol addiction cannot be settled. The basal values were normally distributed. Within each group standard deviations and means were of the same order of magnitude.

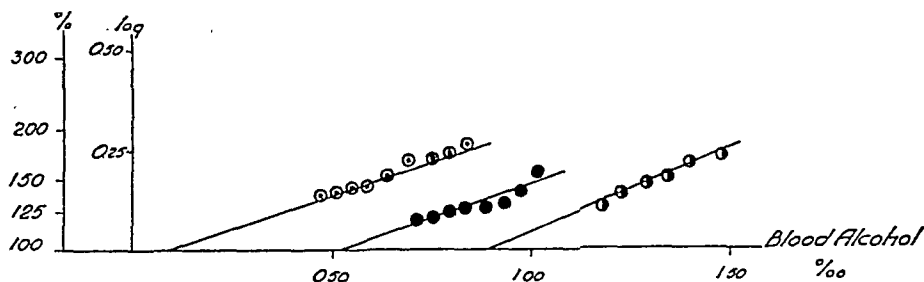


Fig. 21. Brightness for fusion.

Log intensity plotted against blood alcohol in abstainers, moderate drinkers and heavy drinkers.

- |                     |                          |
|---------------------|--------------------------|
| ○ Abstainers        | (mean of 8 experiments). |
| ● Moderate drinkers | > > 16 >                 |
| ◐ Heavy             | > > 8 >                  |

After alcohol ingestion the same principal findings were noted in the three groups: an increase of the brightness for fusion, accompanying the rising part of the blood alcohol curve, a maximum approximately coinciding with the time of the blood

alcohol maximum, and a decrease of the symptoms with the decreasing part of the alcohol curve. If the degree of disturbance due to alcohol be referred to the prevailing blood alcohol curve a straight relationship during the decreasing phase was found for all groups (fig. 21). However, a striking difference can be noticed. The disappearance thresholds for the symptoms occurred at different blood alcohol levels, the lowest being in abstainers, the highest in heavy drinkers; the differences in blood alcohol level were significant (table 11).

Can these relative differences in the values for the disappearance thresholds for the symptoms be explained by the different dosages in the three groups of subjects? If this were the case one would expect a correlation between the disappearance thresholds and the dosages ( $c_0$ ). But no such correlation was found. Moreover, a lowering of the blood alcohol level in one and the same subject as, for instance, by food, did not alter the relationship between symptoms and blood alcohol (see fig. 20).

It might moreover be said that a different level of basal values was the cause of the difference, but the following discredits this. (a) The level of the basal values was identical in the group of abstainers and that of moderate drinkers, nevertheless the average thresholds of the two groups differed. (b) A lower level would indicate an impaired function. This would mean, according to that theory of habituation which assumes compensation of "familiar" functions, that the group of habituals would yield poorer results, as they showed poorer basal values, which would indicate their being less familiar with that special function than the other groups. In reality they were the least intoxicated, so that there is no relationship between basal value and degree of intoxication to compare with the influence of habituation.

A real difference exists therefore with regard to the reaction to alcohol between abstainers, moderate drinkers and heavy drinkers, *i. e.* there is a real *habituation* to alcohol (see definition p. 95). The mechanism of this habituation is not explained by any changes in alcohol absorption, ratio of distribution or rate of combustion but is localized to the central nervous system, the retina being a central nervous structure.

The process consists in an increase of the threshold for the alcohol effect with increasing degree of habituation.

## 2. Sensitivity of the Cornea.

The basal values of the three groups were only comparable for the February series — 25 experiments in all. They showed no difference from each other (table 10).

The changes after alcohol ingestion, when plotted against time, were principally the same in all groups: an increase of the pressure necessary to elicit the blinking reflex by air stimulation, parallel to the blood alcohol curve. When the log differences were plotted against blood alcohol, two characteristics were noted:

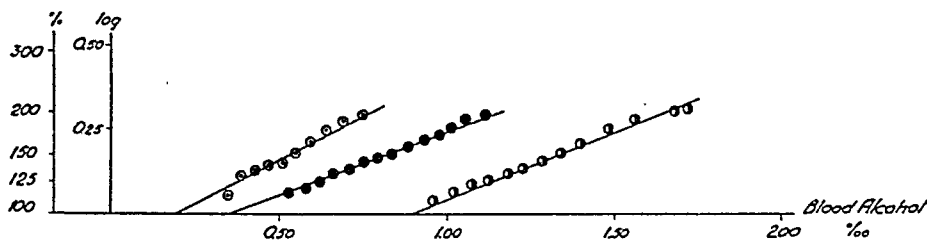


Fig. 22. Corneal sensitivity.

- |   |                   |                           |
|---|-------------------|---------------------------|
| ○ | Abstainers        | (mean of 10 experiments). |
| ● | Moderate drinkers | › › 14 ›                  |
| ◐ | Heavy             | › › 10 ›                  |

1. The approximate linear relationship between the degree of symptoms, expressed as the logarithm of the pressure in mm Hg, and the blood alcohol concentration. This was found in all three groups (fig. 22).

2. With regard to the threshold values, however, they differed significantly. The threshold was 0.20 ‰ for the group of abstainers, 0.36 ‰ for the moderate drinkers, and 0.90 ‰ for the heavy drinkers, the differences being statistically significant (table 11).

These experiments clearly show the process of habituation to be an increase of the disappearance threshold with an increasing degree of habituation. Whether there are any compensating mechanisms causing this rise in threshold or whether this is only due to a changed reaction of the nerve cells, *i. e.* an increased tolerance to alcohol, is difficult to establish; the evidence favours the latter assumption as far as this special function is concerned.

Table 10.  
*Survey of Basal Values in Abstainers, Moderate Drinkers and Heavy Drinkers.*

T e s t	Abstainers			Moderate Drinkers			Heavy Drinkers			Probability (P) that the Groups coincide
	Number of indi- viduals	Basal Values		Number of Indi- viduals	Basal Values		Number of Indi- viduals	Basal Values		
		Log	Abs. Value		Log	Abs. Value		Log	Abs. Value	
1	2	3	4	5	6	7	8	9	10	
Flicker test. . . . .	10	2.800	—	17	2.606	—	8	2.190	—	0.01—0.001
Corneal test . . . . .	4	1.418	29.8 mm Hg	7	1.168	30.6 mm Hg	7	1.517	36.4 mm Hg	> 0.2
Stand. Stead. (ord. Rb)	10	2.473	297.2 mm <sup>2</sup>	17	2.461	281.1 mm <sup>2</sup>	10	2.512	325.1 mm <sup>2</sup>	> 0.2
„ „ (mod. Rb)	10	2.825	668.3 mm <sup>2</sup>	17	2.846	701.4 mm <sup>2</sup>	10	3.157	1371 mm <sup>2</sup>	> 0.2
Finger-Finger test . .	10	2.330	220.9 mm <sup>2</sup>	17	2.317	210.6 mm <sup>2</sup>	10	2.239	180.8 mm <sup>2</sup>	0.2—0.05
Subtraction test . . .	10	0.567—1	0.392 min	17	0.611—1	0.431 min	10	0.588—1	0.541 min	> 0.2
Bourdon test (BC) . .	10	2.122	133.7 Letters	17	2.079	122.2 Letters	10	2.001	102.3 Letters	0.01—0.001
„ „ (BT) . . . . .	10	2.158	142.1 Letters	17	2.139	159.5 Letters	10	2.068	119.1 Letters	0.05—0.01

## B. Motor Functions.

### 1. Standing Steadiness.

The same basal values were found for all three groups for the ordinary Romberg position as well as for the modified (table 10). The standard deviations of all groups also were of the same magnitude, indicating these groups to be homogeneous with regard to variability.

Alcohol ingestion induced the same changes as those already described in the normal material: an increase in the area of sway with rising blood alcohol, the maximum of effect coinciding approximately with the height of the blood alcohol, a decrease parallel to the falling part of the blood alcohol and a return to normal before the alcohol.

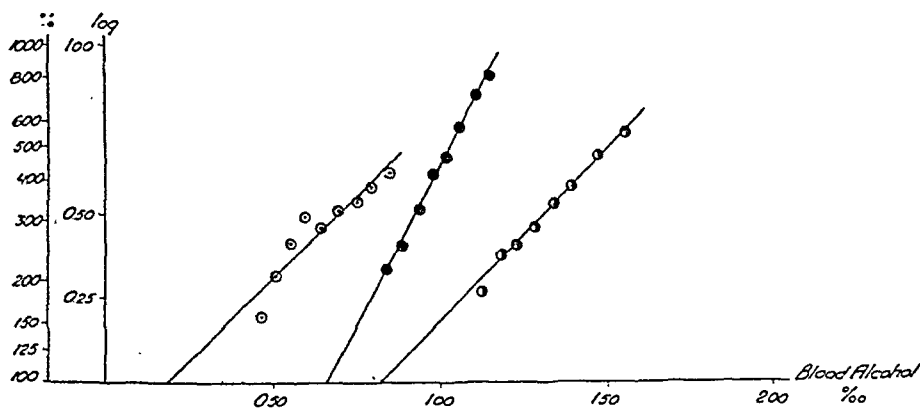


Fig. 23. Standing steadiness (ord. Romberg position).

- Abstainers (mean of 10 experiments).
- Moderate drinkers , , 17 ,
- Heavy , , 10 ,

Referring the logarithmic difference (p. 16) to blood alcohol gave approximately straight lines (fig. 23, 24). The disappearance threshold was lower for the abstainers and higher for the heavy drinkers than it was for the moderate ones. The slopes of these regression lines, too, indicated a difference between the three groups, the abstainers being somewhat more quickly intoxicated with rising blood alcohol than the heavy drinkers (table 11).

These results agree with those found in animals by NEWMAN *et al.* (1936 a, b, 1937 a, b, 1938); they reported that after habituation the impairment in neuro-muscular in-coordination, as judged from a staggering gait, inability to go upstairs, etc., was less than in normal animals. A psychic compensation as part of the mechanism of habituation could not be excluded.

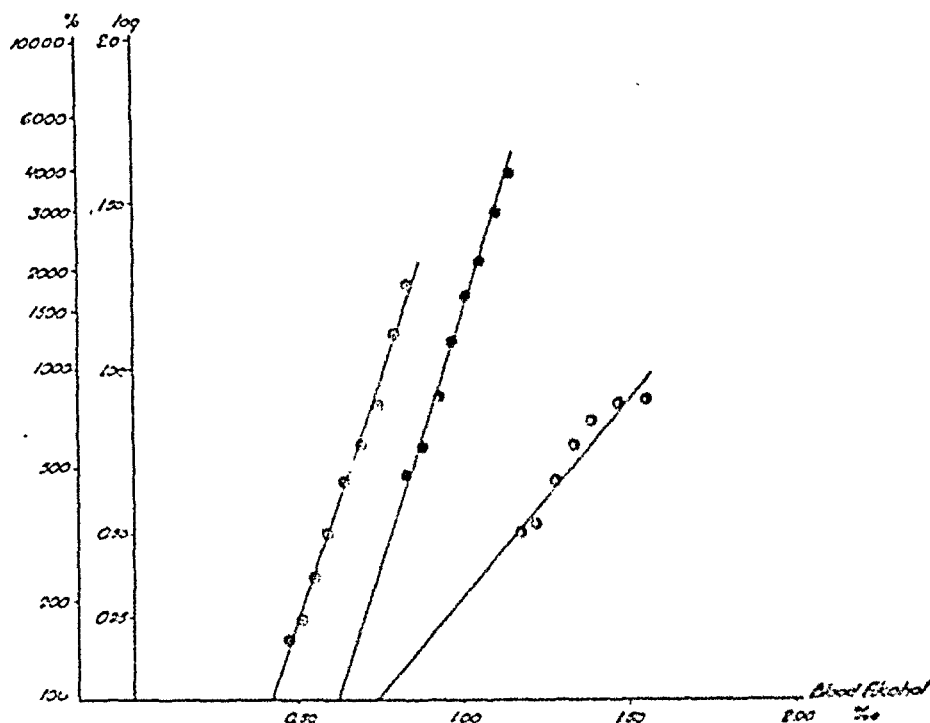


Fig. 24. Standing steadiness (mod. Romberg position).

- Abstainers (mean of 10 experiments).  
 ● Moderate drinkers , , 17 ,  
 ○ Heavy , , 10 ,

## 2. Finger-Finger Test.

The mean basal values of the three groups are given in table 10. The slight differences between the groups are not significant but may suggest a tendency to greater skill in the group of heavy drinkers, which is composed of workmen (p. 13). The changes

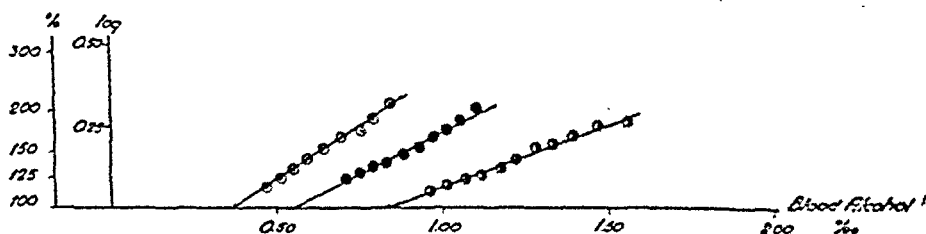


Fig. 25. Finger-finger test.

- Abstainers (mean of 10 experiments).  
 ● Moderate drinkers , , 17 ,  
 ○ Heavy , , 10 ,

after alcohol ingestion were principally the same when plotted against time. But when plotted against blood alcohol (fig. 25), they differed from each other: the disappearance thresholds (table 11) were lower for the abstainers as compared with moderates and higher for the heavy drinkers.

## C. Psychological Functions.

### 1. Subtraction Test.

The mean basal values of the three groups were 0.40, 0.46 and 0.54 min, the differences not being significant (table 10). It might be assumed that this test is largely influenced by mathematical ability and education. In such a case the group of heavy drinkers would show a decidedly poorer performance than the one really observed. The fact that the mean values do not differ significantly from each other will instead be explained by other psychological

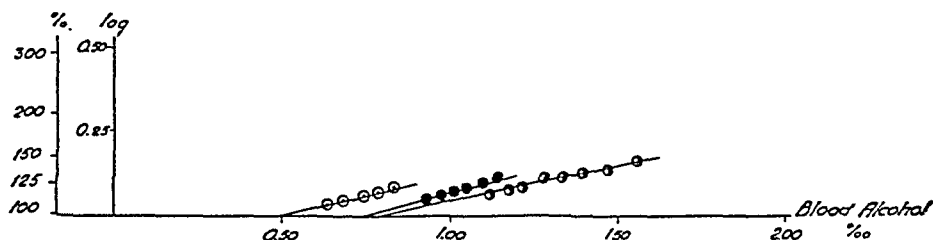


Fig. 26. Subtraction test.

- |                     |                           |
|---------------------|---------------------------|
| ○ Abstainers        | (mean of 10 experiments). |
| ● Moderate drinkers | , , 17 ,                  |
| ◐ Heavy             | , , 10 ,                  |

factors playing a relatively higher rôle than education, *i. e.* primarily the amount of psychic inhibition. The inhibiting processes tend to prolong the total time.

Alcohol ingestion tends to impair the performance of the test in all groups. Referring the degree of disturbance to blood alcohol (fig. 26) gave the same principal findings as with other tests: a rectilinear relationship and a different threshold value. The difference is smaller than with the other tests. This is partly due to a greater ability of the moderate group to compensate for the effect of alcohol (table 11).

### 2. Bourdon Test.

The basal values for the three groups are seen in table 10, the difference between abstainers and moderate drinkers on

Table 11.

*Thresholds and Slopes of Tests after Alcohol Ingestion in 10 Abstainers, 17 Moderate Drinkers and 10 Heavy Drinkers.*  
 From Average Regression Lines between log Symptoms and Blood Alcohol Concentration.

T o s t	Disappearance Threshold			S l o p e				Probability (P) that the groups coincide	
	Abstainers %	Moderate Drinkers %	Heavy Drinkers %	Abstainers Log	Moderate Drinkers Log	Heavy Drinkers Log	Threshold	Slope	Z
	1	2	3	4	5	6	7		
Flicker test . . . . .	0.103	0.529	0.900	0.3617	0.5536	0.1261	< 0.001	> 0.2	
Corneal test . . . . .	0.201	0.563	0.904	0.5432	0.3948	0.4245	< 0.001	< 0.001	
Stand Stand. (ord. Rb)	0.186	0.659	0.819	0.9936	1.9251	1.0283	< 0.001	< 0.001	
"  (mod. Rb)	0.418	0.623	0.714	2.9023	3.0656	1.2084	< 0.001	< 0.001	
Finger-Finger test . .	0.371	0.556	0.839	0.6591	0.5203	0.3917	< 0.001	< 0.001	
Subtraction test . . .	0.503	0.748	0.786	0.2361	0.2755	0.2133	< 0.001	0.05—0.01	
Bourdon test (BC) . .	0.440	0.702	0.860	0.3427	0.4485	0.3328	< 0.001	0.05—0.01	
"  (BT) . . .	0.396	0.722	0.865	0.3034	0.3719	0.2873	< 0.001	0.2 — 0.05	



the one hand, and heavy drinkers on the other being significant. This may be due to the different educational standard of the three groups (p. 13). The lower mean of the heavy drinkers might be an expression for their lower standard of education if there

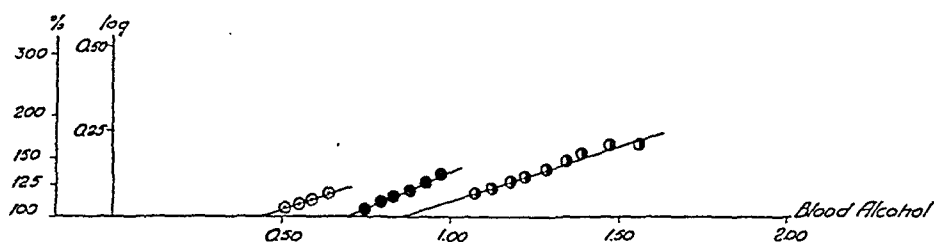


Fig. 27. Bourdon test (number of letters correctly marked).

- |   |                   |                           |
|---|-------------------|---------------------------|
| ○ | Abstainers        | (mean of 10 experiments). |
| ● | Moderate drinkers | › › 17 ›                  |
| ◐ | Heavy             | › › 10 ›                  |

would be a positive correlation between the Bourdon test and education.

Alcohol ingestion induced the changes already described in normal individuals: a poorer performance with the rising part of the blood alcohol, a maximum of disturbance occurring earlier than the blood alcohol maximum, a more or less horizontal part for one hour or two and a decreasing part parallel to that of the blood alcohol and reaching the basal value before the alcohol returned to normal. Referring the decreasing part of the symptoms

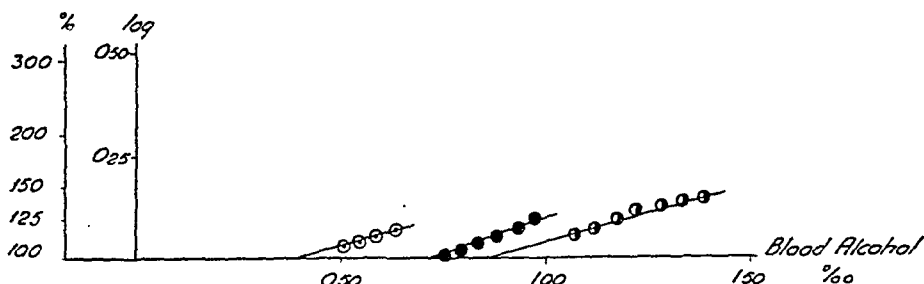


Fig. 28. Bourdon test (total number of letters marked).

- |   |                   |                           |
|---|-------------------|---------------------------|
| ○ | Abstainers        | (mean of 10 experiments). |
| ● | Moderate drinkers | › › 17 ›                  |
| ◐ | Heavy             | › › 10 ›                  |

to the blood alcohol during its post-absorptive period gave an approximate straight relationship for all three groups. The threshold values (figs. 27, 28 and table 11) differed significantly from each other. No difference was seen in the slopes.

An objection against the interpretation of the results found might be that the method of evaluation might exaggerate the divergence found to the advantage of the heavy drinkers. The fact is, however, that the plotting of logarithmic differences against blood alcohol rather diminishes than exaggerates this divergence. The logarithmic difference, which corresponds to the percentage increase, is larger with small basal values, as is the case in the heavy drinkers, and tends to give a higher curve, when plotted against time. When plotted against blood alcohol it tends to give a steeper slope. In spite of this a divergence was clearly seen. The experiments suggest a higher disappearance threshold even in a function, where normally the habituals give poorer results, as indicated by their lower average basal value.

One theory of habituation assumes that phenomenon to be due to a psychological compensation in the habituated individual to the influence of alcohol, primarily in functions familiar to him. These experiments clearly indicate that if such a compensating mechanism exists it is as well to be seen in functions not familiar to the habituated individual, as the basal values were different in the three groups tested.

---

## Conclusions.

The findings common to all functions tested on abstainers, moderate drinkers and heavy drinkers, were:

The general course of intoxication was the same in all groups, when plotted against time, and the average curves, given for a normal material could as well serve to illustrate the course in the other groups, too.

Referring the logarithmic differences (p. 16) to blood alcohol during the decreasing part gave another pattern. The curves of all groups (figs. 21—28) were approximately rectilinear, confirming the principal findings in the normal material. But they differed from one another with respect to two features (table 11): the disappearance thresholds differed significantly ( $P < 0.001$ ), and for the corneal, ordinary ROMBERG, modified ROMBERG and finger-finger test the slope of the curves differed significantly, too ( $P < 0.001$ ). The disappearance thresholds were illustrated in fig. 29. A number of conclusions can be drawn from the results presented.

The general trend of all the functions tested was that the disappearance thresholds varied parallel to the degree of habituation. The difference between the *individual* thresholds in the three groups was largest for the sensory functions, considerable for motor functions, and least for the subtraction test. These findings were not correlated with changes in the basal values (table 10), as the heavy drinkers showed a somewhat lower basal value for flicker and BOURDON test and an insignificant tendency to lower values of the finger-finger test.

For the abstainers and the moderate drinkers the thresholds were lower for sensory and higher for psychological functions as compared with motor functions. In the heavy drinkers no changes of that kind were seen: the thresholds were rather uniform with a tendency to lower values for motor functions. It is tentative to interpret these findings in the following manner.

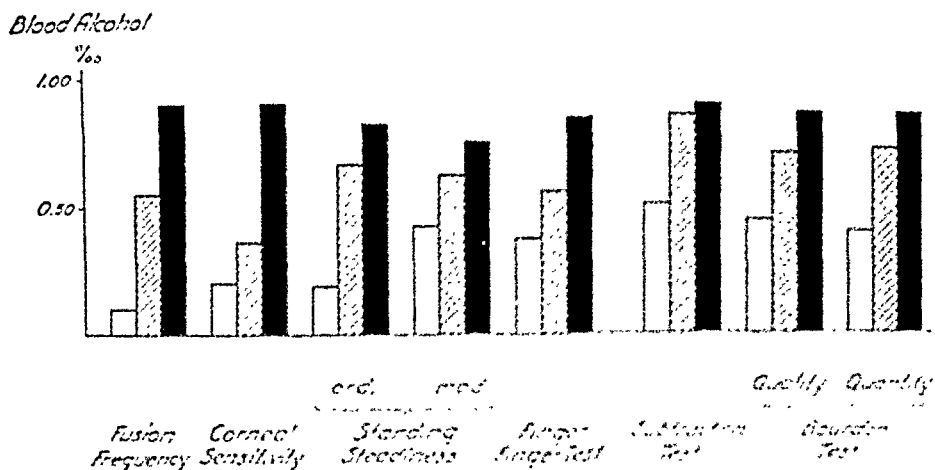


Fig. 29. The influence of habituation on the decrease threshold.

- Abstainers.
- ▨ Moderate drinkers.
- Heavy drinkers.

Habituation primarily involved a shift of the disappearance thresholds to higher values, which was proved by the consistent change in all of them.

The average disappearance thresholds for all the functions tested were within the group of abstainers 0.33 ‰, within the moderates 0.61 ‰ and within the heavy drinkers 0.86 ‰ (mean of cols. 1, 2, 3, resp., of table 11).

As to the presence of a possible compensation, such a mechanism is not altogether excluded. A compensation in normal individuals was suggested under certain conditions. ANDRESEN (1938) and others assume habituation to involve a compensation of "familiar" functions. This would imply moderate drinkers to compensate symptoms to a higher degree than abstainers and *e. g.* account for the large difference in disappearance thresholds between abstainers and moderates in the ordinary ROMBERG and the subtraction test (fig. 29). It would at the same time explain the slight difference between the moderate and the heavy drinkers with regard to the subtraction test, such an operation not being "familiar" to the heavy drinkers. — On the other hand the theory of compensation does not explain the large difference between moderates and heavy drinkers with regard to other functions, *e. g.* the corneal test, where no differences were found in basal values, or in the flicker test, where the heavy drinkers even had lower basal values than the other groups. Thus the change in threshold

seems to account for the greatest part of the difference in degree of intoxication between abstainers, moderates and heavy drinkers.

With regard to the slopes of the curves in figs. 21—28, their general trend suggested a lowered slope in heavy drinkers. If this assumption holds good, it would suggest a slower rate of action of alcohol on the central nervous system in this group.

To illustrate the differing degree of intoxication of the three groups at a given blood alcohol concentration, fig. 30 is given.

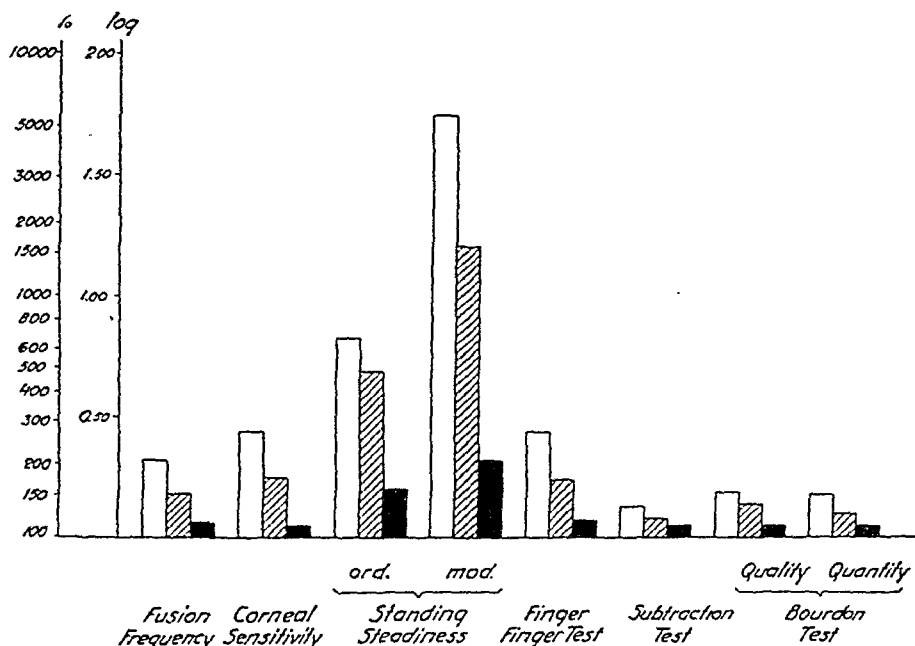


Fig. 30. The degree of intoxication in abstainers, moderate drinkers and heavy drinkers at a blood alcohol concentration of 1 ‰.

- Abstainers.
- ▨ Moderate drinkers.
- Heavy drinkers.

The degree of disturbance at a blood alcohol concentration of 1 ‰ was calculated from the regression lines in figs. 21—28.

The enormous differences between the groups as compared with the shift in thresholds already stated is due to two factors: the logarithmic relationship between degree of intoxication and blood alcohol, and the slight tendency to lower slopes for the curves of the heavy drinkers as compared with the others.

It can thus be concluded that there really exists a habituation to ethyl alcohol pharmacologically, implying a changed reaction to

the prolonged use of alcohol, habituated individuals being less, and not habituated individuals being more influenced by the same dose.

About the nature of habituation it is now ascertained that changes in the rate of absorption, in the distribution ratio or in the rate of disappearance of alcohol from the blood *cannot* explain the difference.

The relationship between degree of intoxication and blood alcohol was changed, primarily causing a shift in the disappearance thresholds to higher values, parallel to the degree of habituation in man. The changes in the slopes of the regression lines representing the approximate rectilinear relationship between log symptoms and blood alcohol, were not consistent, but suggested a tendency to a less steep slope with increasing degree of habituation.

### **Tolerance and Resistance.**

Evidence is now gathering that a varying tolerance to alcohol exists. It was shown that there existed a relationship between the logarithm of a symptom and blood alcohol in a single individual, and likewise in homogenous groups of individuals with respect to alcohol habits. The disappearance thresholds were graphically computed (table 12), and showed within all groups departures from each other, larger than the experimental error. This means that a varying tolerance to alcohol exists within groups of individuals with similar alcohol habits, due to random variation (p. 84).

The differences between the groups are due to habituation, as they are of the same order of magnitude as those computed from average curves (table 11).

Changes in the blood alcohol level, due to changes in the course of alcohol in the body thus imply changes in the degree of intoxication. The principal factor governing the degree of intoxication or alcohol tolerance is the height of the blood alcohol level. A slow absorption of alcohol with a subsequent lowering of blood alcohol, or a depression of the whole curve due to the taking of alcohol with a meal, has a great influence on the degree of intoxication, and a rapid absorption with a high blood alcohol maximum causes an increased degree of intoxication.

The changes in degree of symptoms exceeded the relative alterations in blood alcohol, due to the logarithmic relationship between intoxication and blood alcohol. These changes were

Table 12.

*Means of Disappearance Thresholds after Alcohol Ingestion in Abstainers, Moderate Drinkers and Heavy Drinkers.*  
(Computed from Individual Regression Lines.)

T e s t	Abstainers			Moderate Drinkers			Heavy Drinkers			Probability that the groups coincide
	Num- ber	Threshold ‰	Stand. Dev. $\sigma$	Num- ber	Threshold ‰	Stand. Dev. $\sigma$	Num- ber	Threshold ‰	Stand. Dev. $\sigma$	
	1	2	3	4	5	6	7	8	9	10
Flicker test . . . .	8	$0.393 \pm 0.0607$	0.1719	14	$0.637 \pm 0.0318$	0.1190	8	$0.911 \pm 0.0656$	0.1856	$< 0.001$
Corneal test . . . .	8	$0.257 \pm 0.0773$	0.2188	15	$0.401 \pm 0.0560$	0.2176	10	$0.879 \pm 0.0770$	0.2440	$< 0.001$
Stand. Stead. (ord. Rb)	8	$0.516 \pm 0.0856$	0.2426	14	$0.686 \pm 0.0409$	0.1531	8	$1.165 \pm 0.0921$	0.2608	$< 0.001$
> (mod. Rb)	10	$0.443 \pm 0.0564$	0.1786	16	$0.590 \pm 0.0726$	0.2905	7	$1.047 \pm 0.0952$	0.2520	$< 0.001$
Finger-Finger test . .	10	$0.375 \pm 0.0672$	0.2127	17	$0.655 \pm 0.0505$	0.2084	10	$0.973 \pm 0.0645$	0.2041	$< 0.001$
Subtraction test . . .	10	$0.571 \pm 0.0824$	0.2608	17	$0.973 \pm 0.0338$	0.1391	10	$1.005 \pm 0.0520$	0.1646	$< 0.001$
Bourdon test (BC) . .	10	$0.597 \pm 0.0720$	0.2307	17	$0.711 \pm 0.0364$	0.1500	10	$0.708 \pm 0.0766$	0.2426	$0.01-0.001$
> (BT) . .	10	$0.565 \pm 0.0822$	0.2598	17	$0.710 \pm 0.0454$	0.1872	10	$0.891 \pm 0.1022$	0.3202	$0.05-0.01$

furthermore not of the same order of magnitude for different functions, the motor functions being most influenced.

On an average an increase of the blood alcohol concentration of 1 ‰ over the threshold value of a function caused a percentage increase of intoxication, the normal being 100 ‰, of 238 ‰ for the sensory functions, 6,890 ‰ for the motor functions, and 232 ‰ for the psychological functions tested.

After elimination of variations in alcohol tolerance due to the changes in blood alcohol level of an individual, by referring the degree of intoxication to blood alcohol, there still remained a varying tolerance within a *homogenous* group of individuals, proved by *e. g.* the varying disappearance thresholds of different individuals (table 12). This is most probably due to the same random variations which cause varying tolerance in general: the hormonal state of the individual (GOLDBERG and STÖRTEBECKER 1943), etc. Between groups of individuals, differing from one another as to their degree of habituation, differences in tolerance were found too. As variations in the blood alcohol level were ruled out by the correlation of the symptoms with blood alcohol, the variation between groups with different alcohol habits is due to *resistance* (p. 8).

At least one of the factors causing varying resistance could be isolated, *viz.* the degree of habituation, and the presence of another was strongly suggested, *viz.* the ability of the organism to compensate psychically the influence of alcohol; some facts could be interpreted in the direction of this ability being increased by habituation.

The significance of the factors which constitute personality (FLEMING 1935), which possibly exerts itself in the varying degree of psychic compensation, were not elucidated in this paper which dealt primarily with alcohol tolerance in its pharmacological implication.

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## Summary.

The course of ethyl alcohol intoxication in man was followed in sixty-one experiments on forty-nine subjects: eleven abstainers, twenty-four moderate and fourteen heavy drinkers, by means of test methods on sensory, motor and psychological functions.

I. *The course of alcohol intoxication.* The experiments were arranged so that the tests, six in all, were performed on every one of the subjects: twice before the ingestion of alcohol to procure a basal value, and then seven times after ingestion to follow the course of intoxication. The alcohol was given in a 40 % solution, 0.63 to 1.42 g alcohol per kg. Blood alcohol samples — double or triple determinations — were taken nine times, and the symptoms referred to blood alcohol.

a. *Sensory functions.* In order to study changes in sensory functions the *flicker* test (GRANIT and HARPER 1930) was used to determine the log brightness for fusion of the eye to intermittent light at a constant flicker frequency, and in some experiments too the critical fusion frequency of the eye at constant brightness.

To determine the *sensitivity of the cornea* a new test was worked out, using a puff of air blown against the cornea as stimulus, and the blinking reflex as criterion of the effect, expressed in mm Hg pressure of the air necessary to elicit the blinking.

b. *Motor functions.* Two quantitative tests were worked out for this purpose: one on *standing steadiness*, where the area of sway of an individual, while standing for 15 seconds, first in an ordinary ROMBERG position and then in a modified, was photographed and measured planimetrically, and as a second, a quantitative *finger-finger test*, where the area covered by fifty dottings was likewise determined planimetrically.

c. *Psychological functions.* Finally a *subtraction* test, where the total time for a series of subtractions was determined, and a BOURDON test with marking of three given letters as many times

as they appeared in a text in four minutes, were employed for testing psychological functions.

The *variability* of the test methods was ascertained on thirty-nine individuals under different conditions; the influence of practice and fatigue was determined on twelve subjects in control experiments without alcohol lasting from six to seven hours.

The degree of intoxication after alcohol ingestion in an individual was found to vary with the blood alcohol concentration. The *relationship* between symptoms and blood alcohol was determined by plotting log symptoms against the concentration of alcohol in the blood during the increasing and decreasing phase of the symptoms, and was shown to be approximately *rectilinear*.

The appearance of the symptoms of intoxication began at a blood alcohol level, termed the *appearance threshold*, which was found within normal material to vary between 0.31 and 0.65 ‰ on an average for the different functions tested. The *disappearance threshold*, defined as the blood alcohol level, at which the symptoms of intoxication disappeared, was under the same conditions found to vary between 0.36 and 0.75 ‰. The appearance threshold was lower than the disappearance one for the modified ROMBERG, the finger-finger and the psychological tests; for the other tests the thresholds had identical values.

The degree of intoxication was observed to be *higher* for the first-mentioned symptoms at a given blood alcohol concentration during the *rising* part of the alcohol curve than during the falling. This was proved to be due primarily to the difference in threshold values, and its connection with *compensation* of symptoms was suggested.

By graphical computation of the disappearance thresholds for one and the same function within a homogeneous group of individuals, the thresholds were shown to be distributed at random, suggesting a *varying* tolerance to alcohol.

II. *The influence of food.* The intake of *food* with the alcohol decreased the subsequent blood alcohol level and lowered the degree of intoxication in all functions tested, if expressed in logarithmic units, proportionately to the depression of the alcohol level. The disappearance thresholds were proved to be of approximately the same magnitude, whether alcohol was taken on an empty stomach or with a meal. This indicates that the lowering

effect that food has on alcohol intoxication is due to its depression of the blood alcohol curve.

III. *The influence of habituation.* The influence of habituation on the degree of intoxication was studied by applying the technique described on groups of abstainers, moderate and heavy drinkers.

A different reaction on the ingestion of alcohol was proved to exist between the three groups for all functions tested: the abstainers being the most intoxicated and the heavy drinkers the least influenced by a given dose. This implies a real habituation to alcohol to exist in the pharmacological sense, and some characteristics were elucidated.

The *blood alcohol* curves of the three groups differed somewhat, suggesting a more rapid absorption of alcohol among the heavy drinkers and a slight difference in the rate of disappearance of alcohol from the blood. These differences could *not* explain the divergence in alcohol tolerance between the groups.

When referring the log symptoms to the blood alcohol concentration, an approximately *straight relationship* was observed for all groups; The relation curves differed significantly in one respect: the disappearance thresholds were lower for the abstainers and higher for the heavy drinkers, as compared with the group of moderates: the average of all functions tested was 0.33 ‰ within the group of abstainers, 0.61 ‰ within the moderates and 0.86 ‰ within the heavy drinkers, the differences being statistically significant.

On account of the experimental evidence presented, habituation to alcohol in man was interpreted as an *increased tolerance*, i. e. a lowered degree of intoxication, and was due to an increase in the blood alcohol threshold for the symptoms, localized to the central nervous system and parallel to the degree of habituation.

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